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LXXXV. THE CARBOHYDRATE OF NORMAL URINE.

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In a recent paper Lund and Wolf [1925] show how the sugar (or sugars) of normal urine can be differentiated from glucose by the fact that, as a result of acting upon the urine in a Barcroft apparatus with a specially active yeast, carbon dioxide was evolved from specimens to which glucose had been added, but not from the urine itself. The first part of the present investigation shows that a very clear distinction may be made between the two by quite simple means.

If the urine is acted upon, in the presence of a small amount of toluene and a low concentration of phosphoric acid, by ordinary baker's yeast, the urine sugar is unaffected, whereas any small amount of added glucose is readily fermented with corresponding diminution in the true sugar content as estimated by Benedict and Osterberg's method [1918]. The investigation has been carried out, in order to simplify conditions, on the urines of persons on a moderate carbohydrate diet. The urinary sugar in cases where large quantities of carbohydrate are taken has not yet been fully examined. The loss of sugar previously ascribed to fermentation by Benedict and Osterberg [1918, 1921], who differentiated between fermentable and unfermentable sugars in normal urine, is evidently due to the action of organisms already present in the specimens or introduced along with the yeast. Indeed it is a well-known fact that the concentration of sugar in normal urine steadily diminishes on standing, without the addition of yeast.

To endeavour to show further differences between the urinary sugar and glucose, the attempt was made to isolate the former in the form of its osazone, and compare this with phenylglucosazone. This derivative was prepared by first treating the urine with Benedict's mercuric nitrate reagent to remove nitrogenous constituents, which seriously interfere with osazone formation, and glycuronic acid, and then applying the osazone-forming reagents in the usual way. The purification of the collected crystals was difficult, leading finally to a product which was evidently not quite chemically pure. It was without definite melting point, gradually decomposing between the temperatures 170–182°, passing into a viscous, tarry mass at 182–184°. Fig. 1 shows

the appearance of the urine sugar osazone when finally crystallised from boiling water, whilst Fig. 2 shows the appearance of phenylglucosazone obtained by an identical procedure. Owing to its marked insolubility in water, it is rather difficult to obtain glucosazone in good crystalline form from this medium, but it will be observed that under the conditions employed a characteristic shape is attained, although the individual crystals are considerably smaller than those usually obtained from dilute alcohol solution or from water containing an excess of phenylhydrazine.

The difference between the two substances is thus shown in most striking manner. Water is demonstrated to be much more suitable for the crystallisation of the urinary sugar osazone than for that of the glucosazone, owing to the greater solubility in hot water of the former. This fact has been used in devising fairly simple clinical laboratory tests, which it is hoped to publish shortly, to determine pathological sugar excretion in doubtful cases.

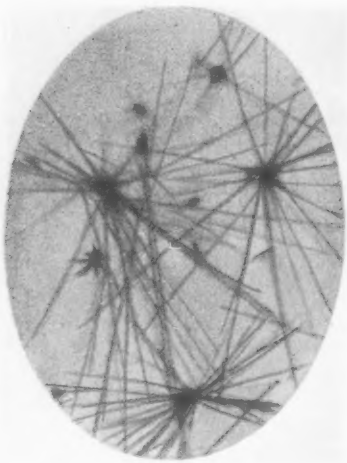

Fig. 1.
(Magnification 175)

Fig. 2.
(Magnification 175)

The elementary analysis of the urinary sugar osazone gave figures closely approximating to those calculated for a hexosazone. Unfortunately it was impossible to obtain a reading of the optical rotation. In attempting to apply the method of Nanji [1923] another difference between this osazone and most others of the sugar group was discovered. The *N* hydrochloric acid used in this method of determining nitrogen converts part of the material into a black friable humus-like mass, and the nitrogen value, obtained by subsequently

estimating the phenylhydrazine split off, was always considerably below that obtained by the combustion method.

In this investigation other forms of osazone have been met with in very small quantities, but in no case has it been possible to obtain any evidence of the presence of glucosazone. However, the compound described above is the derivative of what is undoubtedly the chief component of the sugars of normal urine.

The ease of decomposition of the osazone described above finds a parallel in the comparative instability of the sugar itself, as illustrated by the finding that, whilst in the majority of urines examined hydrolysis with hydrochloric acid (2 parts of concentrated acid to 100 parts of urine by volume) leads to an increase in the reducing sugar content through hydrolysis of dextrin-like carbohydrate, in not a few instances the reverse effect is obtained, indicating a destruction of the reducing sugar and the absence of hydrolysable dextrin to compensate. Where treatment with acid has led to an increase in free sugar, that portion liberated is for the most part fermentable under the same conditions as is glucose. In such cases the constant value attained after fermentation is at times below the initial before hydrolysis, again pointing to the partial destruction of the original reducing sugar.

Experimental.

All sugar estimations were made by Benedict and Osterberg's colorimetric method [1918].

Fermentation of normal urine.

To 100 cc. of urine were added 10 drops of toluene, 0·25 cc. of 85 % phosphoric acid and 20 drops of a thick suspension in saline of ordinary baker's yeast. Specimens were incubated at 37°.

Specimen	Before fermentation Sugar as glucose %	Time of fermentation hours	After fermentation Sugar as glucose %
1	0·090	24	0·087
2	0·032	24	0·033
3	0·040	17	0·035
4	0·100	17	0·102
5	0·090	17	0·084

Fermentation with added glucose.

0·1 g. of pure glucose was added to 100 cc. of urine which was then treated in the manner already described.

Specimen	Before fermentation Sugar as glucose %	Time of fermentation hours	After fermentation Sugar as glucose %
1	0·131	5	0·024
2	0·191	5	0·094
3	0·177	4	0·111

Isolation of the osazone.

Successive portions of 75 cc. of urine were treated exactly according to Benedict's technique for removing nitrogenous constituents and glycuronic acid, and in each case 100 cc. of clear colourless filtrate were collected after the separation of the excess zinc dust. This quantity was heated for 1½ hours in a boiling water-bath with 2 g. phenylhydrazine hydrochloride, 6 g. sodium acetate, and 2 cc. glacial acetic acid. On allowing to stand overnight, crystals of osazone separated out and were collected from the several portions. In all 5 l. urine were treated in this manner and 1 g. crude osazone obtained. The crude product was crystallised twice from dilute aqueous alcohol, then twice from dilute pyridine and, finally, three times from hot water. For this purpose it was found most convenient to adopt a procedure somewhat similar to Soxhlet extraction, except that, instead of using the mid-piece of the Soxhlet apparatus, the thimble containing the substance was supported in the extraction flask above the level of the solvent in such a manner that the drops from the condenser fell upon the crystals at a temperature approaching 100° and filtration went on simultaneously with the hot extraction. Acicular crystals separated immediately on cooling.

The most highly purified material gave the following analytical figures:

(1)	C 61·1 %	H 6·3 %	N 15·3 %
(2)	60·9	6·3	15·5
A hexosazone requires	60·33	6·4	15·64

Fermentation of urine after hydrolysis.

Hydrolysis was performed with 2 parts concentrated hydrochloric acid per 100 parts of urine by volume for 15 mins. at 100° and fermentation carried out as described above.

(*a*) Hydrolysis increasing the reducing sugar content.

Specimen	Initial sugar (as glucose) %	Sugar (as glucose) after hydrolysis %	Sugar (as glucose) after hydrolysis and fermentation %
1	0·040	0·045	0·035
2	0·014	0·035	0·010
3	0·032	0·089	0·030
4	0·083	0·150	0·103
5	0·039	0·050	0·012

(*b*) Hydrolysis decreasing the reducing sugar content.

Specimen	Initial sugar (as glucose) %	Sugar (as glucose) after hydrolysis %	Sugar (as glucose) after hydrolysis and fermentation %
1	0·121	0·093	0·077
2	0·046	0·024	0·014

SUMMARY.

1. Under conditions in which glucose is readily fermentable the sugar of normal urine is completely unfermentable.

2. The osazone of the chief urinary sugar has been isolated, and marked differences between it and glucosazone noted with regard to crystalline form, melting point, solubility and stability towards acids.

3. The hydrolysis of the complex carbohydrate frequently found in normal urine liberates a reducing sugar which is fermentable.

The above investigation was carried out with the aid of a grant from the Government Grant Committee of the Royal Society, to whom the author tenders his warmest thanks.

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LXXXVI. THE HEMICELLULOSES. PART IV.

THE HEMICELLULOSES OF BEECH WOOD.

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A REPORT TO THE FOREST PRODUCTS RESEARCH BOARD, DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH.

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In a previous communication by the author [1923] it was pointed out that the term "hemicellulose," as defined by Schulze [1892], was applied to a class of substances soluble in alkalis, from which solution they could be precipitated by acids or alcohol, and much less resistant to the action of hydrolysing agents than cellulose.

The investigation described in this paper indicates that the hemicelluloses are not as originally thought true carbohydrates (pentosans, hexosans, etc.) but contain acid groups (glycuronic and galacturonic acids) and are, therefore, more nearly allied to the pectic substances than to cellulose.

The hemicelluloses from different sources, furthermore, have been found to vary to a considerable extent, especially as regards the products of hydrolysis, and, indeed, two hemicelluloses have been obtained from beech wood itself, for on adding a large excess of acid to a sufficiently concentrated alkaline extract of the hemicellulose it is possible to obtain a precipitate, the clear filtrate from which gives on the addition of two volumes of alcohol (95 %) a second precipitate. These two precipitates are essentially different both in appearance and properties. That which is directly precipitated by acid is more resistant to hydrolysis, giving, after several hours' treatment with dilute sulphuric acid, xylose and apparently 11 % glycuronic acid. On treatment with 12 % hydrochloric acid by the method of Tollens 50 % of furfural is obtained.

The second precipitate, on the other hand, seems to be still more closely allied to the pectic substances. It gives, on hydrolysis, apparently about 63 % of galacturonic acid together with some arabinose and a small amount of galactose. The amount of furfural obtained is 42·7 %. This precipitate also yields small amounts of mucic acid on oxidation with nitric acid.

The amount of the second precipitate obtained from beech wood is small in comparison with that obtained by direct precipitation with acid. Workers

in these laboratories on the hemicellulose of non-lignified tissue find, however, that the opposite is the case in the substances which they have examined. They also have obtained two hemicelluloses of which that not precipitated directly by acid is in predominant quantity.

The whole subject of the relation between the hemicelluloses and the pectic substances in lignified and unlignified tissue seems to open up a very fruitful field for investigation. The pectic substances occur in unlignified tissue in much greater amounts than the hemicelluloses [Norris and Schryver, 1925]; in lignified tissue the opposite is found to be the case [O'Dwyer, 1925].

The obtaining of sufficient quantities of the "uronic" acids for the purpose of identification has been tedious work owing to their decomposition during the course of hydrolysis. However, the method of estimating the carbon dioxide given off in such cases by treatment with 12 % hydrochloric acid, advanced by Nanji, Paton and Ling [1925], has proved very helpful as showing the existence of these substances as products of hydrolysis of the hemicellulose of beech wood, and in giving the amounts of these acids which might be obtained from each precipitate if their decomposition during hydrolysis could be avoided.

Before the separation of the two precipitates had been effected, a sugar acid was obtained after hydrolysis of the mixed precipitate from sawdust 93 years old with 1 % sulphuric acid. When, however, hemicellulose from beech wood 130 years old was treated in the same manner, apparently no sugar acid was present in the hydrolysed solution. It was thought at the time that the age of the wood was responsible for its non-appearance, but further experiments showed that, while in the earlier stages of hydrolysis a sugar acid appeared which gave a barium salt soluble in water, hydrolysis for a further period produced an acid giving an insoluble barium salt. When the hydrolysis was carried still further both acids apparently disappeared, as after the addition of the amount of barium hydroxide necessary for the neutralisation of the sulphuric acid the solution was no longer acid.

These results were extremely puzzling, and it was only after repeated experiments on the preparation and hydrolysis of hemicellulose had been carried out, that it was found that the material originally employed was a mixture of two hemicelluloses, one of which could be obtained from the alkaline solution by direct precipitation with acid, while the other separated out from the acid filtrate on addition of alcohol. These two substances were then isolated in as pure a condition as possible, and, when they were subjected to hydrolysis under the same conditions as before, it was found that the sugar acid giving an insoluble barium salt was a product of hydrolysis of the hemicellulose directly precipitated by acid, whilst that forming a soluble barium salt came from the second precipitate, which, as before stated, is less resistant to hydrolysis. They were subsequently found to be respectively glycuronic and galacturonic acid. The isolation of the acid depends therefore on the conditions of hydrolysis and not on the age of the tree, although experiments

showed that age has apparently some bearing on the amount of each hemicellulose obtained.

Norris and Schryver [1925] have isolated two hemicelluloses from non-lignified tissue of a single plant material (turnips). One of these is water-soluble and the other is precipitated from the soda extract by acid. Their work will be made the subject of a further communication.

Preparation of the hemicelluloses.

Separation of two hemicelluloses. 4500 g. of sawdust from beech wood 80 years old were used in the preparation of these hemicelluloses, and an endeavour was made to obtain as pure a product as possible. After thorough extraction with hot water, the sawdust was treated for a short time with 0·2 % sodium hydroxide in order to remove the small amount of protein (0·7 to 0·8 %) previously found present by Kjeldahl's method. This weak alkaline solution did not apparently affect the hemicelluloses which were subsequently extracted with 4 % sodium hydroxide. Lime water was then added to the soda extract, to bring down any pectic acid present, and the mixture filtered. It was found possible, by adding a sufficient excess of glacial acetic acid to the filtrate, to effect a complete separation of the precipitate which had previously been obtained in a colloidal condition. The clear filtrate obtained after the removal of this precipitate was concentrated, and two volumes of 95 % alcohol were added. A second precipitation now occurred. These two precipitates were also obtained from a soda extract of beech sawdust which had previously been treated with hot 0·5 % ammonium oxalate.

The hemicellulose obtained by direct precipitation with acetic acid was now washed with small quantities of distilled water until the filtrate became colourless, dissolved again in 4 % sodium hydroxide and re-precipitated with acid. It was then electro-dialysed for 10 days in order to effect the removal of sodium acetate and other inorganic matter which might be present. After treatment with graded strengths of alcohol and dry ether, it was finally dried to constant weight in a vacuum desiccator over phosphorus pentoxide. The yield was a little over 5 % of the weight of the sawdust.

The second hemicellulose, precipitated from the acid filtrate by the addition of alcohol, which was soluble in water, was found on dialysis to pass through the membrane of the dialyser to some extent. It was accordingly, after filtering, re-dissolved in water, the solution concentrated and the hemicellulose re-precipitated with alcohol. After repeating this process, a substance was obtained, which, after drying with graded strengths of alcohol and then with dry ether, was found to contain only a small amount of inorganic matter. This product was dried to constant weight in a vacuum desiccator over phosphorus pentoxide. The yield was about 1 % of the weight of the sawdust.

Properties of the two hemicelluloses. The hemicellulose obtained by direct precipitation of the soda extract with acetic acid is an amorphous substance, almost white in colour, insoluble in dilute acids and alcohol and soluble in

alkalis. When freshly prepared, it is dispersed by water to a small extent, but after drying is insoluble in cold water. It forms, on treatment with hot water and cooling, a somewhat gelatinous mass. A greenish coloration is obtained with iodine. It does not reduce Fehling's solution, and is laevo-rotatory $[\alpha]_D = -107°$, the determination being made in a 1 % sodium hydroxide solution. The furfural yield, obtained by Tollens' method, reckoned on the ash-free substance was 50·0 %. The ash amounted to 4·0 % and was found to be mostly ferric oxide.

The following figures were obtained by combustion, the results being given for the ash-free substance:

(*a*) 0·1605 g. gave 0·2615 g. CO_2 and 0·1218 g. H_2O.
C = 44·43 %, H = 8·43 %, O = 47·47 %.

(*b*) 0·1415 g. gave 0·2288 g. CO_2 and 0·1025 g. H_2O.
C = 44·10 %, H = 8·05 %, O = 47·85 %.

On oxidation with nitric acid (sp. gr. 1·15) by the method of Tollens [Norris and Schryver, 1925] no mucic acid was obtained.

In order to ascertain the relation between the time of hydrolysis and the reducing sugars present, 0·5 g. of the substance was hydrolysed with 1 % sulphuric acid for different periods.

Bertrand's method was employed to ascertain the reducing power of the hydrolysed solution in each case.

Duration of hydrolysis (hours)	Reducing sugar as glucose (%)
1	20·5
2	45·3
4	62·5
6	86·5
8	88·7

The hemicellulose brought down from the acid filtrate by alcohol is amorphous, dark in colour, and is, as before stated, soluble in water. It is also dissolved by dilute acids and alkalis but is insoluble in alcohol and ether. On the addition of iodine, a slight decoloration of the iodine solution was observed.

The substance does not reduce Fehling's solution and has a specific rotation in 0·5 % sodium hydroxide of − 120°. The furfural yield is 42·7 %.

5·84 % of ash was present and consisted largely of ferric oxide, with a small amount of carbonates.

The following figures were obtained by combustion of the ash-free substance:

(*a*) 0·2400 g. gave 0·3843 g. CO_2 and 0·1570 g. H_2O.
C = 43·66 %, H = 7·27 %, O = 49·07 %.

(*b*) 0·2410 g. gave 0·3890 g. CO_2 and 0·1568 g. H_2O.
C = 44·10 %, H = 7·23 %, O = 48·67 %.

(The author is indebted to Mr B. D. Wakeford for the combustion figures and also for those for ash and furfural.)

The mucic acid estimation gave the following result:

0·5388 g. gave 4·40 % mucic acid ≡ 4·80 % galactan.
0·4950 g. gave 4·30 % mucic acid ≡ 4·76 % galactan.

The relation between the time of hydrolysis and the amount of reducing sugars was ascertained as before, and the following figures were obtained:

After 1 hour's hydrolysis ...	28·5 %
,, 2 hours' ,, ...	60·0 %
,, 4 ,, ,, ...	74·2 %
,, 6 ,, ,, ...	80·5 %

It is proposed to call the hemicellulose obtained by direct precipitation with acid hemicellulose A, and that obtained by adding alcohol to the acid filtrate, hemicellulose B.

Hydrolysis of the two hemicelluloses.

(*a*) *Determination of carbon dioxide evolved on treatment with strong acid.*

As carbon dioxide is given off when hemicellulose is submitted to hydrolysis by acids, the method adopted by Nanji, Paton and Ling [1925] for its estimation was followed. Accordingly estimations were made on the acid and alcohol precipitates of hemicellulose just described, and also on a hemicellulose containing both precipitates. Hemicellulose from which the pectic substances had not been removed was also used in these experiments, as it was expected that the percentages of "uronic" acids would be slightly higher in such cases.

In each experiment carbon dioxide was given off, and the following results have been obtained:

Hemicellulose (description of)	Amount taken (g.)	% CO_2 on decarboxylation	% "uronic" acids (calc.)
(*a*) Mixed precipitate containing pectic substances	0·5849	3·31	13·24
	0·6500	3·52	14·08
(*b*) Mixed precipitate free from pectic substances	0·5676	3·00	12·0
	0·4977	2·91	11·64
(*c*) Acid precipitate free from pectic substances	0·4207	2·74	10·96
	0·4130	2·76	11·04
(*d*) Alcohol precipitate free from pectic substances	0·4705	15·98	63·92
	0·4590	16·02	64·08

From the above results it is evident that the alcohol precipitate gives a large amount of carbon dioxide on decarboxylation, and resembles pectic acid in this respect. The slight differences between the yields of carbon dioxide from hemicellulose free from, and that containing, pectic substances are accounted for by the small percentage of such substances in beech wood. The yield of carbon dioxide from the acid precipitate shows again that this substance is quite unlike that brought down by alcohol.

(*b*) *Hydrolysis with dilute sulphuric acid.*

The following hydrolyses were now carried out on the two hemicelluloses:

(i) 15·5 g. of hemicellulose A were hydrolysed for 2½ hours with 200 cc. of 1 % sulphuric acid. After adding the requisite amount of baryta to neutralise the sulphuric acid, the hydrolysed solution was no longer acid, so apparently no sugar acid is present.

(ii) 11·8 g. of the same hemicellulose were hydrolysed for 6 hours with 150 cc. of 2½ % sulphuric acid. The hydrolysed solution after the addition of the requisite amount of baryta was found still strongly acid. On the addition of more baryta a salt insoluble in water was obtained. After washing and drying to constant weight, the amount of the salt was found to be 0·1480 g.

(iii) 10·7 g. of the same material were hydrolysed for 5 hours with 1 % sulphuric acid. A very small amount of the insoluble barium salt was obtained.

(iv) 3·74 g. of hemicellulose B were treated with 200 cc. of 1 % sulphuric acid for 2½ hours. After adding baryta as before, the solution was still acid, but, on the addition of more baryta, no further precipitation occurred and a barium salt soluble in water was obtained.

(v) 4·52 g. of the second hemicellulose were hydrolysed for 4½ hours with 200 cc. of 1 % sulphuric acid. The solution was still acid after adding baryta and a soluble barium salt was again formed.

From these results it appears evident that the acid which gives a barium salt insoluble in water is a hydrolysis product of the hemicellulose directly precipitated by acid, or hemicellulose A, while the barium salt which is soluble in water is obtained from a sugar acid produced on hydrolysis from hemicellulose B. The insoluble barium salt was in each case filtered off, washed, and dried to constant weight. The yield from the various hydrolyses was 5·3527 g.

The barium salt which is soluble in water was separated from the sugars present in the solution in the manner described at a later stage.

(*c*) *Identification of the sugars produced by the hydrolysis of the two hemicelluloses.*

Hemicellulose A. The filtrates which were obtained after precipitation of the insoluble barium salt were concentrated under reduced pressure, taken up with hot alcohol, and the solution again concentrated. The syrup thus obtained was boiled under a reflux condenser, evaporated to dryness under reduced pressure, taken up with a little hot alcohol, and the solution again evaporated down and left to dry in a vacuum desiccator over sulphuric acid.

The amorphous mass thus obtained was treated with absolute alcohol under a reflux condenser and filtered while hot. This process was repeated several times, and it was found that the greater part of the sugar present came out of the solution on cooling. It proved to be practically pure xylose, giving a melting point of 148–150° and a specific rotation of + 18°. Bertrand's reagent [1891] was employed as a further means of identification for xylose.

To 1 g. of the sugar, 1·5 g. of bromine and 3 g. of cadmium carbonate were added, and the characteristic crystals of the double cadmium salt of xylose $(C_5H_9O_6)_2Cd + CdBr_2 + H_2O$ obtained.

From a third portion of the sugar *l*-xylose diformal $C_5H_6O_5(CH_2)_2$ was obtained [de Bruyn and Van Eckenstein, 1903].

The residue obtained after treatment with hot absolute alcohol was dissolved in hot 80 % alcohol. Crystals separated out on cooling, and, after drying, 1 g. was treated with 2 g. of phenylhydrazine hydrochloride and 3 g. sodium acetate in a test tube in a boiling water-bath [Mulliken, 1905]. An osazone appeared after 10 minutes' heating. It consisted of an orange-yellow mass, with oily drops at the surface. Under the microscope, the crystals appeared to be those of the osazone of xylose. They gave a melting point of 160° on recrystallising from 50 % alcohol.

As oily drops appearing on the surface of an osazone are an indication of the presence of arabinose, an attempt was made to obtain the diphenylhydrazone by treating a little of the sugar with an excess of diphenylhydrazine in alcoholic solution [Neuberg, 1900, 1904]. When the hydrazone had separated out it was dried and weighed. The crystals melted at 199° but a very small amount of the diphenylhydrazone, less than 1 %, was obtained. As the amount of arabinose present is so small it may be regarded as an impurity, probably from an incomplete separation of the two hemicelluloses. As will be seen later, it occurs in fairly large quantities as a product of the hydrolysis of the other hemicellulose.

Hemicellulose B. As this hemicellulose gives on hydrolysis a soluble barium salt, the hydrolysed solution was concentrated under reduced pressure and evaporated to dryness. It was then extracted successively with absolute, 80 % and 60 % alcohol. A precipitate was obtained on the cooling of the filtrate in each instance, but the bulk of the sugar was dissolved by 80 % alcohol, a very small amount being obtained from absolute alcohol, whilst the residue from 60 % alcohol proved to be a little of the barium salt itself with a trace of sugar.

The sugar obtained in this case by solution in hot absolute alcohol was found to be arabinose. It melted at 160°, but, owing to the small amount obtained, the specific rotation could not be taken. Treatment with diphenylhydrazine gave the diphenylhydrazone of arabinose, melting at 202°.

A mixture of sugars was obtained from the hot 80 % alcohol solution. This gave a melting point of 143–145° and a specific rotation of + 95° ($c = 4$ %). The diphenylhydrazone of arabinose was again obtained and also the phenylhydrazone of galactose. To obtain this 1 g. of the mixture was treated with a slight excess of phenylhydrazine in the cold. After a short time colourless crystals separated out. They were dissolved in a very little water and recrystallised from a small amount of absolute alcohol; M.P. 156°. Galactose phenylhydrazone melts at 158°. The following table shows the amounts of sugar extracted by various strengths of alcohol from the hydrolysis products.

Hemicellulose A.

11·8 g. taken for hydrolysis.

Concentration of alcohol	Sugar g.	Percentage on hemicellulose
(*a*) Absolute	4·220	35·79
(*b*) 80 %	2·6885	22·78
(*c*) 60 %	Nil	—

Hemicellulose B.

7·5 taken for hydrolysis.

(*a*) Absolute	0·4959	6·80
(*b*) 80 %	1·8984	25·0
(*c*) 60 %	Trace	—

(*d*) *Examination of the sugar acids.*

Hemicellulose A. This, as stated before, yielded a barium salt insoluble in water. It was found that 0·6188 g. of the barium salt contained 0·0342 g. of barium sulphate and that, after allowing for this, the barium content was 27·0 %. As barium glycuronate contains theoretically 26·19 % of barium, it was decided to add sufficient sulphuric acid to decompose the remainder of the salt and obtain the free acid, if possible. The mixture was then filtered free from barium sulphate and the filtrate concentrated under reduced pressure and finally left for some time in a vacuum desiccator over sulphuric acid. On drying, a substance with M.P. 160–165° was obtained. These figures approximate to the melting point of glycuronic acid and it was decided to try and obtain the cinchonine salt, which had been used by both Neuberg [1900] and Schwalbe and Feldtmann [1925] as a means of identifying glycuronic acid. This was obtained in the form of white needles, melting at 201° and having a specific rotation of + 139°. The cinchonine salt of glycuronic acid melts at 204° and has a specific rotation of + 138·6°.

As a further means of identification a little of the acid was dissolved in water and treated with 5 g. of *p*-bromophenylhydrazine and an equal amount of glacial acetic acid [Neuberg, 1899]. A product was obtained, which after recrystallisation from 60 % alcohol melted at 234° and had a specific rotation of − 365° when dissolved in a mixture of 6 cc. absolute alcohol and 4 cc. pyridine. The *p*-bromophenylhydrazide (or hydrazone) of glycuronic acid, according to Neuberg [1899], melts at 236° and has a specific rotation of − 369°. There is therefore no doubt that the acid hydrolysis product of hemicellulose A is glycuronic acid.

Hemicellulose B. This hemicellulose yields, as before stated, a barium salt soluble in water. An estimation of the barium present gave a figure a good deal lower than the theoretical amount of barium in barium galacturonate. It was found that a little sugar was also present. This was dissolved in 80 % hot alcohol, and the examination of the salt proceeded with. The cinchonine salt was prepared, as in the case of glycuronic acid, and was found to agree closely with that obtained by Ehrlich [1917] from galacturonic acid. It was more

soluble than the salt obtained from glycuronic acid. Crystals were obtained by evaporating to dryness and taking up with a very little water. They melted at 157° and had a specific rotation of + 137°. According to Ehrlich, the cinchonine salt of galacturonic acid melts at 158° and has a specific rotation of + 134°.

The naphthoresorcinol test [Tollens, 1908] was carried out on each of these acid solutions by heating with a little naphthoresorcinol in the presence of an equal volume of hydrochloric acid (sp. gr. 1·19). A bluish, turbid solution was obtained in each case, and on treating the deposit with ether, a violet blue coloration appeared. There is little doubt that the acid obtained on hydrolysis of hemicellulose B is galacturonic acid. Owing to the small amount of this acid (it being remembered that the yield of hemicellulose B is only a small one), a more complete examination was not possible.

Summary.

Hemicelluloses are not, as originally thought, true carbohydrates (hexosans, pentosans, etc.) but contain acid groups.

Two hemicelluloses have been isolated from beech wood, each giving different physical and chemical properties. One can be directly precipitated by acid, hemicellulose A, and the other, hemicellulose B, is obtained on precipitation from the acid filtrate by alcohol.

Hemicellulose A yields, on hydrolysis, xylose and an amount of carbon dioxide corresponding to 11 % of glycuronic acid, whereas hemicellulose B yields arabinose and an amount of carbon dioxide corresponding to 63 % of galacturonic acid, together with small amounts of galactose.

The acids were obtained only in small amounts, as they decompose during the hydrolysis. Their presence or absence amongst the hydrolysis products was found to be due not to differences in the age of the wood, method of drying, etc., but was determined solely by the method of hydrolysis employed. Similar mixtures of hemicelluloses have been obtained from non-lignified tissue by workers in these laboratories.

The author's best thanks are due to Professor S. B. Schryver under whose direction this work was carried out.

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LXXXVII. ON THE SOLUBILITY OF SOME PICRATES AND THE DETERMINATION OF GUANIDINES IN URINE.

By ISIDOR GREENWALD.

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(*Received May 21st, 1926.*)

In a paper published about a year ago, Sharpe [1925] refers to certain criticisms by the present writer [Greenwald, 1924] of the methods employed by Burns and Sharpe [1917], Findlay and Sharpe [1920] and Nattrass and Sharpe [1921] for the detection and determination of guanidines in urine. Essentially, these were that creatine might be oxidised to methylguanidine by the mercuric salts employed by Burns and Sharpe and that the method employed by Findlay and Sharpe and by Nattrass and Sharpe made no adequate provision for the separation of creatinine and of ammonium salts. In order to meet this criticism, Sharpe proposed a slight modification of the method previously employed. The urine (250 cc.) is treated, in succession, with tannic acid, $Ba(OH)_2$, H_2SO_4 and $BaCO_3$, filtering off each precipitate as produced. The final filtrate is evaporated to a syrup and extracted with absolute alcohol. The extract is evaporated and the residue then obtained is extracted with 20 cc. alcohol. The alcohol is then evaporated and the residue is dissolved in 10 cc. water. After adding 10 cc. of a saturated aqueous solution of picric acid, the mixture is set aside for crystallisation. Guanidines, if present, should crystallise out as the picrates, whereas creatinine should remain in solution.

Although proposed as a method for the determination of *guanidines*, the experimental data presented refer only to the isolation of *guanidine*. It seems to be assumed that the same method will be applicable to the determination of methylguanidine and of dimethylguanidine. That the picrates of these substances might be more soluble than is guanidine picrate seems not to have been considered.

Even guanidine picrate, as was shown by Medes [1925], is not sufficiently less soluble than creatinine picrate to permit of a satisfactory separation of the former from an excess of the latter. If the quantity of guanidine picrate is less than one-third of the amount of creatinine picrate, such crystals as separate must contain creatinine picrate. Only if at least 20 mg. of guanidine are present for each 113 mg. of creatinine is it at all possible for pure guanidine picrate to separate from a solution containing the two picrates.

With methylguanidine and dimethylguanidine, the separation is hopeless. As is indicated in the table, the solubilities of the picrates of creatinine, methylguanidine and dimethylguanidine, at both 10° and 20°, are almost

The diphthalic ester acids are suitable for fractionating the liquid portion of the unsaponifiable substance, as shown by the following experiment: 5 g. of the liquid part of the unsaponifiable fraction were dissolved in 25 cc. of pyridine, 10 g. of phthalic anhydride were added, and the solution boiled for 1 hour under reflux, after which the solution was poured into 400 cc. of warm water. By the addition of some more pyridine almost all was dissolved. After cooling the solution was filtered and the filtrate was washed with 250 cc. of ether. The ester acids passed into the ether as pyridine salts. (A sample of the solution gave no precipitate on the addition of H_2SO_4 in excess.) The ethereal solution of pyridine salts was repeatedly washed in dilute H_2SO_4 and then once or twice with water. The ether solution, which was reddish-brown, was dried with Na_2SO_4, filtered and evaporated, yielding a residue of 7·34 g. The equivalent weight of the preparation was found to be 383. Calculated for the selachyl monophthalic ester acid 478; for the selachyl diphthalic ester acid 311.

Thus there was probably present a mixture of mono- and di-phthalic ester acids, and the esterification had by no means been complete.

A portion of this preparation was dissolved in dry ether and several volumes of light petroleum (30°–50°) were added, whereby a considerable quantity of dark-coloured, viscous substance was precipitated. The petroleum layer, which after standing became quite clear, left after evaporation 1·569 g. of brown viscous oil, the equivalent weight of which was 507. The dark, viscous, precipitated substance was shaken up two or three times with light petroleum, dissolved in ether, and the ether evaporated. The weight of the residue was 3·155 g. and its equivalent weight 341. It thus appeared that it is possible to separate mono- and di-phthalic ester acids by the aid of their different solubilities in light petroleum. Thus even if complete esterification is not achieved it will be possible to arrive at pure preparations of diphthalic ester acids by precipitating with light petroleum. The solution in light petroleum will, in addition to monophthalic ester acids, contain any hydrocarbons, glycerides and cholesterol phthalic ester acids which might be present.

If we wish to isolate the hydrocarbons, we may remove the ester acids from the light petroleum solution by washing with alkali, or we can dissolve the fraction in alcohol and precipitate the phthalic ester acids as Pb salts by the addition of an alcoholic solution of Pb acetate. The oil of *Somniosus microcephalus* contained very little hydrocarbon, since only 70 mg. could be isolated from 20 g. liquid unsaponifiable substance. After the bulk of the cholesterol had been removed by crystallisation from an alcoholic solution, the unsaponifiable substance of cod-liver oil contained about 9 % of hydrocarbons.

The acid phthalic ester of cholesterol is prepared by boiling a solution of 1 g. of cholesterol and 2 g. of phthalic anhydride in 5 cc. of pyridine for 1 hour. The mixture is poured into water and the precipitated ester acid is removed by filtration. In contrast to the selachyl diphthalic ester acids, the pyridine

salt does not appear to exist in a watery solution. The precipitate is treated with dilute HCl and recrystallised twice from alcohol, whereby white crystals are obtained, which melt at 161–161·5°. The equivalent weight was 539, calculated 534. The cholesterol phthalic ester acid is easily soluble in ether, but is not precipitated by light petroleum, even when considerable quantities of the latter are added. In this way it is possible to free our preparations from cholesterol without resorting to digitonin, the high price of which renders its use difficult in sufficiently large quantities.

Batyl diphthalic ester acid. 2 g. of batyl alcohol and 4 g. of phthalic anhydride, dissolved in 25 cc. of pyridine, were allowed to stand for $2\frac{1}{2}$ days at room temperature. After diluting with water the solution was washed repeatedly with ether. After washing with dilute sulphuric acid and finally with water, the ethereal solution was dried over Na_2SO_4, filtered and evaporated. The residue was 3·332 g. of a firm, white substance, which melted at 91°. It was dissolved in ether and precipitated with light petroleum. After this had been repeated twice, the melting point was constant at 97°. The equivalent weight was 312·2; calculated for $C_{20}H_{40}O_3(C_8H_5O_3)_2$, 312.

Preparation of selachyl alcohol by the aid of salts of the diphthalic ester acid.

We did not succeed in producing selachyl diphthalic ester acid in crystalline form. It always forms jellies when separating from its solutions.

Attempts were therefore made to use various salts for preparation of the pure phthalic ester acid. Lead and copper salts appeared to be best suited for this purpose. The potassium, sodium and magnesium salts are soluble in water.

The lead salt of selachyl diphthalic ester acid. 3 g. of potassium salt were dissolved in 25 cc. of water and precipitated with 3 g. of neutral lead acetate, which also was dissolved in 25 cc. of water. The precipitate, after being dried over sulphuric acid in a vacuum desiccator, weighed 3·2 g. It was almost completely soluble in ether and was again precipitated by adding alcohol. After twice precipitating in this way, the analysis gave the following results:

0·2292 g. salt gave 0·0856 g. $PbSO_4$. Found 25·51 % Pb, calculated 24·91 % Pb.

The lead salt was decomposed by shaking an ethereal solution with dilute hydrochloric acid and then with water. The phthalic ester acid thus isolated had the iodine number 42·4; calculated 40·7. If the conditions were altered, a Pb salt of another composition was precipitated. If an alcoholic solution of the potassium salt of the ester acid was precipitated by an alcoholic solution of lead acetate, a precipitate was obtained which, after being thrice dissolved in ether and precipitated by alcohol, showed a constant melting point of 140°. 0·2024 g. of this gave 0·0904 g. of $PbSO_4$, corresponding to 30·57 % Pb. From this was produced the free ester acid and from the latter the free alcohol. In a dose of 0·01 mg. daily this was found to produce growth in young rats, the

growth of which had been checked on account of lack of vitamin A. The active substance, however, is evidently only precipitated by alcohol along with the Pb salt. The precipitate was amorphous, and the ethereal solution had colloidal characters. It also appeared (see below) that the preparation of selachyl alcohol obtained from the crystalline copper salt had neither growth-promoting nor antirachitic properties.

Preparation of the copper salt. 16·9 g. of the phthalic ester acid with equivalent weight 320, *i.e.* fairly pure diphthalic ester acid, were dissolved in 75 cc. of alcohol and neutralised by means of 160 cc. of 0·3304 *N* NaOH. To the solution thus obtained were added 17 g. of crystalline copper sulphate, dissolved in 100 cc. of water. The precipitate thus obtained, which weighed 18 g., was almost completely soluble in 650 cc. of boiling alcohol, and on cooling crystallised out. After recrystallising several times, 0·0986 g. gave 0·0114 g. CuO, corresponding to 9·24 % Cu; calculated for the copper salt of selachyl-diphthalic ester acid, 9·25 % Cu.

The free ester acid is produced by passing H_2S into a boiling alcoholic solution of the copper salt. CuS is precipitated colloidally, but is brought to an easily filtrable form by shaking with Fuller's earth. The free ester acid is a very viscous, faintly yellowish oil, which showed no signs of crystallisation after standing.

The free alcohol was produced by saponification of the ester acid with alcoholic potash and subsequent solution in ether. The ether was evaporated with water and left a faintly yellowish, readily flowing oil.

7·745 mg. gave 20·647 mg. of CO_2, and 8·691 mg. of H_2O, corresponding to 72·73 % C and 12·56 % H. Calculated for $C_{20}H_{40}O_3$, 73·07 % C and 12·30 % H.

This preparation of selachyl alcohol showed by animal experiments in daily doses of 0·16 mg. neither growth-promoting nor antirachitic properties, and moreover did not give the well-known colour reaction with concentrated sulphuric acid, either alone or after the addition of cholesterol.

Preliminary remarks regarding the unsaponifiable fraction of cod-liver oil.

It is intended to investigate the unsaponifiable fraction of cod-liver oil on the lines indicated above. Some preliminary experiments have given the results that this fraction (prepared in the same manner as that described for shark oil), after the cholesterol had as far as possible been removed by crystallisation from alcohol, still contained 14·3 % of cholesterol. The acetyl number was 222·3, the iodine number 154·2 and there was about 9 % of hydrocarbons present. Drummond, Channon and Coward found *ca.* 8 % spinacene. Correcting the figures found for acetyl and iodine numbers according to these data, we obtain for the alcohols present an acetyl number of 265 and iodine number 144. The acetyl number of the alcohols should indicate that there are present compounds similar to those in shark oil, but the high iodine number indicates that they must be more unsaturated than selachyl

alcohol. Orienting experiments have further shown that the alcohols of cod-liver oil by esterification with phthalic anhydride in pyridine behaved analogously to selachyl alcohol, forming diester acids, insoluble in light petroleum. Their equivalent weight was 314.

Of the salts, that of barium appeared to be best suited for the preparation of the pure acid, for it was fairly easily soluble in boiling alcohol and separated out on cooling. These investigations are being continued by the present writer.

SUMMARY.

1. The alcohols present in the unsaponifiable fraction of the liver oil of *Somniosus microcephalus* can be converted into phthalic ester acids, which may be used for the isolation of the alcohols.

2. The bulk of the alcohols in the oil of *Somniosus microcephalus*, and presumably also of the alcohols in cod-liver oil, are dihydric.

3. The properties of batyl alcohol and selachyl alcohol produced from the oil of *Somniosus microcephalus* agree with the statements of Tsujimoto and Toyama.

4. In the pure condition these alcohols have neither growth-promoting nor antirachitic properties, and do not give the well-known colour reaction with sulphuric acid.

5. The ester acids of the alcohols which are present in cod-liver oil exhibit a close resemblance to selachyl diphthalic ester acid. The alcohols of cod-liver oil, however, are more unsaturated than selachyl alcohol.

The author is indebted to Professor E. Poulsson for the interest he has taken in this work, and for useful criticism, advice and continuous encouragement.

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XCI. STUDIES OF THE BACTERIAL DECOMPOSITION OF TEXTILE FIBRES.

IV. NOTE ON THE ACTION OF *B. SUBTILIS* AND *B. MESENTERICUS* ON CELLULOSE.

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(*Received June 21st, 1926.*)

Trotman and Sutton [1924] reported that they had found *B. subtilis* and *B. mesentericus* capable of decomposing the cellulose of cotton hairs.

Their work was repeated at this laboratory shortly after its publication without any evidence being obtained in support of their statements [Thaysen and Bunker, 1924]. It was realised, however, that Trotman and Sutton might have been using strains of these organisms in their experiments which, for some reason or other, possessed special properties. An endeavour was therefore made to obtain subcultures from the original strains used by Trotman and Sutton. This was finally made possible through the kind offices of the curator of the National Collection of Type Cultures, Lister Institute.

With these cultures three series of experiments were carried out for the study of their action on cellulose. The series were planned to observe any changes occurring, as follows:

(1) a visible resolution of the cellulose such as takes place when the carbohydrate is decomposed under aerobic conditions;

(2) changes in the appearance under the microscope of the exposed fibres or hairs;

(3) losses in weight of the exposed samples of cellulose.

For the purpose of the first series of experiments the strains were inoculated into a sterile nutrient solution[1] contained in test tubes in which strips of filter paper were partly submerged.

[1] The nutrient solution had the following composition:

Dipotassium hydrogen phosphate	0·1 %
Ammonium sulphate	0·2 %
Magnesium sulphate	0·1 %
Sodium chloride	0·1 %
Peptone	0·1 %
Manganese sulphate, Ferric chloride	one drop each of a 1 % solution per litre

In the second series the fibres of the exposed filter paper as well as exposed cotton hairs were examined microscopically by the swelling test.

In the third series weighed quantities of filter paper contained in a nutrient solution, the whole having been previously sterilised, were inoculated with cultures of the test organism. The loss in weight of the filter paper was determined quantitatively at the end of the experiment.

In the first series the inoculated test tubes were incubated at 37°, in some cases for as long as 55 days. Though a vigorous development of the test organisms took place, the strips of filter paper showed no signs of destruction or disintegration except in two cases, when, after 21 days' exposure, the inoculated paper disintegrated at the place where it emerged from the solution. Attempts to reproduce this phenomenon in subcultures using part of the disintegrated paper as inoculant failed completely though the subcultures were incubated for 30 days or more.

It is interesting to note that the disintegrated fibres examined by the swelling test were absolutely normal, that is, could not be distinguished in any way from normal fibres.

In the second series of experiments, in which samples of filter paper and cotton hairs were examined by the swelling test after exposure for 55 days to the action of *B. subtilis* and *B. mesentericus* the exposed fibres and hairs were in all cases perfectly normal, showing no signs whatsoever of microbiological decay.

In the third series eight flasks containing weighed quantities of filter paper damped with nutrient solution were sterilised. Two of these flasks were left uninoculated to serve as controls. Of the remaining six, three were inoculated with a culture of *B. subtilis*, three with one of *B. mesentericus*. All the eight flasks were incubated at 37° for 28 days.

At the end of this time the filter paper in each flask was washed with hot distilled water, with a 1 % hydrochloric acid solution to remove any calcium carbonate present and again with hot distilled water. After drying, the various samples of paper were weighed. The data relating to these weighings are given in Table I.

Table I.

Flask no.	Inoculant	Weight of paper		Loss of weight %
		Before (g.)	After (g.)	
1	None (control)	12·7999	12·7191	0·63
2	,,	13·8102	13·7603	0·36
3	*B. subtilis*	13·5961	13·4668	0·95
4	,,	12·5762	12·5520	0·19
5	,,	12·5059	12·1979	2·46
6	*B. mesentericus*	12·3832	12·3514	0·26
7	,,	11·1551	11·1832	0·25
8	,,	13·8918	13·5595	2·39

In only two cases—samples 5 and 8—was there a noticeable decrease in the weight of the filter paper after exposure beyond that observed in the control samples. But even here the loss in weight can hardly be claimed to

have passed the limit of accidental experimental errors. To assume that the loss recorded indicates a destruction of the cellulose by the test cultures is entirely unjustified. Had these types been capable of decomposing cellulose it is extremely unlikely that the destruction would have been limited to an occasional removal of 2 % of the total quantity available.

Conclusions.

The three series of experiments reported on in the preceding pages confirm the now generally accepted view that neither B. subtilis *nor* B. mesentericus *is capable of decomposing cellulose.*

The above experiments arose from work carried out on behalf of the Fabrics Co-Ordinating Research Committee of the Department of Scientific and Industrial Research. The authors desire to express their thanks to the Department of Scientific and Industrial Research and to the Admiralty for permission to publish the results obtained.

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XCII. OBSERVATIONS ON THE PREPARATION, PROPERTIES AND SOURCE OF THE PARATHYROID HORMONE. PART I.

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(*Received June 23rd, 1926.*)

The isolation of a parathyroid hormone has been described by Collip and his co-workers [Collip, 1925, 1, 2; Collip, Clark and Scott, 1925; Collip and Clark, 1925, 2, 3, 4]. Their method of extracting the active principle consists in digesting the glands with 5 % hydrochloric acid at 100° for a time not exceeding 1 hour, and the final product is obtained by a series of isoelectric precipitations from the digest fluid. When injected subcutaneously into dogs, it was found that the serum calcium content could be raised. To obtain the maximum effect, Collip recommended injection at four-hourly intervals for a period of at least 24 hours. He also stated that herbivorous animals were resistant to the action of the hormone, a statement supported by the recent observations of Macleod and Taylor [1925].

Experimental.

Variation in the serum calcium content of rabbits.

Owing to the difficulties, such as accommodation, etc., in using dogs for these experiments, it was decided to re-investigate the response of rabbits to the hormone. Large animals of 3 kg. weight or over were employed, and they were fed on cabbage and bran mash throughout the experiments. In the first place it was necessary to investigate the accuracy of methods of determining the serum calcium, and in the second place to work out the normal variations in the serum calcium content of rabbits throughout the 24 hours. By means of Collip and Clark's [1925, 1] modification of Kramer and Tisdall's process, it was found that the serum calcium content could be easily estimated to an accuracy of approximately $\pm$ 2 %. To determine the second point, a series of rabbits were bled at four-hourly intervals throughout the 24 hours, and the changes in their serum calcium contents were followed. In addition, the effects of injecting normal saline and distilled water at four-hourly intervals were controlled. In Fig. 1 five typical curves are reproduced, and it can be seen that the serum calcium content does not vary more than about 1 mg. per

100 cc. during the whole time of the experiment. In all, 15 control animals have been studied, and the rise has never been over 1·5 mg. per 100 cc., but in a few instances the figures have fallen more or less steadily as much as 1·5 mg. per 100 cc.

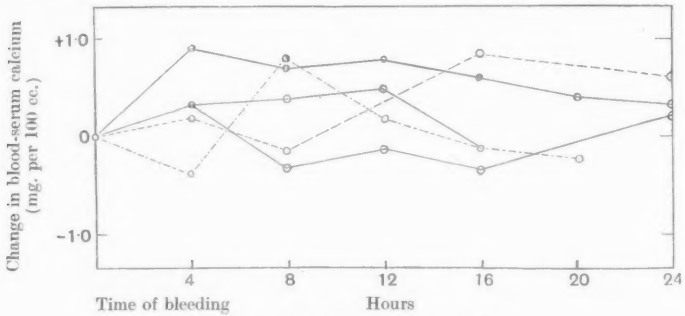

Fig. 1. Variations in normal rabbits.

It was therefore decided that any increase over 1·5 mg. per 100 cc. in the serum calcium content must be regarded as being due to external factors, such as the injection of tissue extracts, and that the conditions of the animal experiment were sufficiently standardised to commence tests upon the activity of various preparations. All solutions employed for this purpose were first tested and found to contain no calcium.

Effect of Collip and Clark's extract upon the serum calcium content.

Despite the statements of previous workers it was felt that, provided sufficient quantities of the hormone were administered to rabbits, there should be an elevation in the serum calcium content. To test this point, a very con-

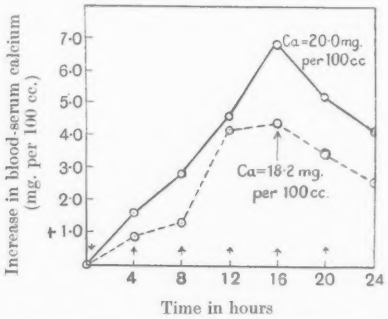

Fig. 2. Parathyroid extract (Collip and Clark).
The arrows indicate time of injection.

centrated extract was made from parathyroid glands by the method of Collip and Clark [1925, 3]. The strength of the final solution was so adjusted that

each injection corresponded to 15 g. of fresh glands. The effects of four-hourly injections for 24 hours on the serum calcium content of two rabbits can be seen in Fig. 2. Although the dose was greatly in excess of that employed by Collip, the observation succeeded in showing that under these conditions rabbits responded to injections of the hormone in a manner exactly similar to that of the dog. An attempt to calibrate these results in terms of dog-units was made by injecting diluted and undiluted "parathormone" (Lilly). This material is standardised according to the method recommended by Collip: each cc. contains 10 dog-units—a dog-unit being one-hundredth part of the amount of hormone required to raise the blood calcium content of a 20 kg. dog by 5 mg. per 100 cc. Injection of this solution into rabbits failed to produce any increase in the serum calcium content, even when 1 cc. was given at four-hourly intervals for 24 hours.

A few experiments were made to determine the minimum quantity of hormone necessary to produce a rise in serum calcium, and, although injections corresponding to 7 g. of gland were found to be effective, it became obvious that a great deal of work would have to be done before any quantitative relationship between the dog- and rabbit-units could be established. The rabbit is therefore much more resistant to the action of the hormone than is the dog, yet, since a uniform rise in serum calcium could always be provoked by the injection of suitable quantities, it was decided to continue the investigation employing rabbits for the test. As already stated, the control experiments had shown that the normal serum calcium content of rabbits never showed a rise of more than 1·5 mg. per 100 cc. throughout the 24 hours, and it was decided to investigate, on a qualitative basis, the nature of substances capable of causing a rise of more than 1·5 mg.

Preparation of the picrate and its properties.

A study of the properties of the hormone suggested that it resembled insulin in constitution: thus its activity is destroyed by pepsin and trypsin, and it gives the common protein reactions [Collip and Clark, 1925, 3]. It was also found to give a strong Pauly reaction. The following experiment also emphasised the similarity to insulin. 49 mg. of the material obtained by Collip and Clark's process were dissolved in 3 cc. of slightly alkaline water, giving a clear straw-coloured solution. 9 cc. of saturated aqueous picric acid solution were added, and the resulting precipitate was collected by centrifugation. The yellow residue was treated with 5 cc. of 70 % acetone, and on stirring it dissolved completely. The acetone was evaporated off and the residue was stirred with 3 cc. of acid alcohol (made by mixing 25 cc. of 3 *N* hydrochloric acid with 75 cc. of absolute alcohol). Ten volumes of acetone were added to the resulting slightly turbid solution, and the white, insulin-like precipitate was allowed to settle. It was then collected and washed with acetone until free from picric acid, and finally with dry ether. The resulting white powder was dried *in vacuo* and was then found to weigh 27 mg. It dissolved

readily in water, giving a clear solution. The potency was tested by injecting three doses of 7 mg. at hourly intervals into rabbits. In Fig. 3 the changes in the blood calcium contents can be followed. This experiment demonstrated that the hormone yielded a picrate which was insoluble in water, but soluble in 70 % acetone, and which could be converted into the hydrochloride by Dudley's method [Dudley and Starling, 1924].

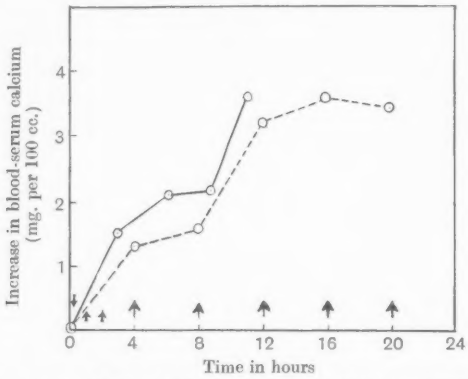

Fig. 3. *Upper curve.* Collip and Clark extract precipitated as picrate and picrate converted into hydrochloride. Injections shown by small arrows.
Lower curve. Extract of parathyroid glands by acetone-picric acid method.

The question at once arose as to whether the hormone could be extracted from the fresh glands by the acetone-picric acid process [Dodds and Dickens, 1924]. 300 g. of glands were extracted by the acetone-picric acid process in exactly the same manner as in the preparation of insulin, and a yield of 405 mg. of hydrochloride was obtained. This was dissolved in water so that 3 cc. of the solution corresponded to 10 g. of glands. The results of administering this quantity at four-hourly intervals can be seen from Fig. 3. The rise in the serum calcium content shows that the hormone can be extracted by this process, without a preliminary acid digestion as in Collip's process. Whether previous treatment with acid would improve the yield by the acetone-picric acid method is at present under investigation.

Effect of insulin and pituitrin upon the blood calcium content.

Since these observations emphasise still more the similarity in the properties of insulin and the parathyroid hormone, it was decided to investigate the action of insulin upon the blood calcium when administered to rabbits under the conditions of the above experiments. Commercial insulin solution, containing 20 units per cc., was diluted 1 in 30 with distilled water and 2 cc. were injected into rabbits in the manner described for the parathyroid hormone. Curve A, Fig. 4, shows the effect upon the serum calcium content, and it can be seen that there is a marked rise. Although the animal was fed throughout the experiment, convulsions occurred twice and their time of appearance is

marked on the chart. The animal was revived with glucose, 5 cc. of a 10 % solution in normal saline being injected intravenously. In order to control the possible effect of glucose injection upon the serum calcium, a rabbit was bled, and 10 cc. of 10 % glucose in normal saline solution were injected intravenously. The rabbit was bled at the end of 4 hours, and the calcium contents of this and of the first specimen were estimated, giving values of 12·1 and 11·9 mg. per 100 cc. respectively. It is safe to conclude, therefore, that this factor could not have influenced the serum calcium curves. 100 g. of fresh pancreas were extracted by the Collip and Clark [1925, 3] process and the resulting material was dissolved in 20 cc. of water to give an opalescent solution. The effect of injecting quantities of 3 cc. of this solution into a rabbit at four-hourly intervals is shown in Curve B, Fig. 4. Insulin, whether made by

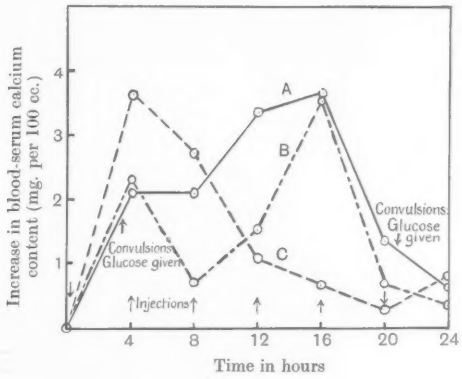

Fig. 4.

Curve A. Commercial insulin.
Curve B. Extract of pancreas made by Collip and Clark's method.
Curve C. Insulin prepared from pancreas by the acetone-picric acid method.

the alcohol or by the acetone-picric acid process is capable of elevating the blood calcium, and the same applies to an extract made from the pancreas by Collip and Clark's process.

The next problem to arise was whether the calcium raising property of insulin was in any way associated with its blood-sugar lowering power, or whether the former property was due merely to a component of the mixture of substances composing insulin. The following experiments were performed with a view to abolishing the blood-sugar lowering action of insulin, and at the same time leaving the calcium raising action intact. Concentrated hydrochloric acid was added to a commercial insulin solution until the concentration of acid was 5 %. The solution was then heated in a boiling water-bath for 1 hour, and after cooling was neutralised. After animal tests, it was found that the blood-sugar lowering activity had been reduced to one-seventh of its original value, and that its calcium elevating power had also been reduced considerably (Curve B, Fig. 5). This experiment unfortunately does not make

it possible to arrive at any decision, since it must be remembered that although the parathyroid hormone can withstand boiling with 5 % hydrochloric acid in the extraction process, the quantity of protein present is sufficient to reduce the hydrogen ion concentration of the mixture very considerably. This factor is not present when purified insulin is treated with 5 % acid, and the conditions are therefore much more severe. Collip has shown that an hour at 100° in 10 % hydrochloric acid is sufficient to destroy the activity of the parathyroid hormone completely.

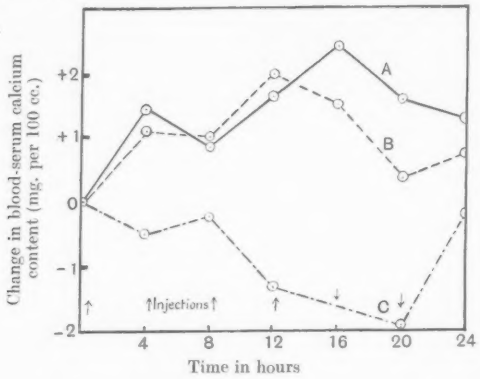

Fig. 5.

Curve A. Pituitrin diluted 1 in 4. Dose: 2 cc.
Curve B. Insulin after treatment with hydrochloric acid.
Curve C. Insulin and pituitrin injected together.

An attempt was made to approach the problem from a pharmacological aspect. It has been shown by Burn [1923] that pituitrin inhibits the blood-sugar lowering action of insulin, and it was thought that if simultaneous injections of insulin and pituitrin were given, interesting effects might be observed on the serum calcium. A commercial solution of pituitrin was diluted 1 in 10. 2 cc. each of these solutions were injected simultaneously into rabbits at four-hourly intervals in the usual manner. The effect on the serum calcium content can be seen in Curve C, Fig. 5. The effect of pituitrin alone is shown in Curve A, Fig. 5.

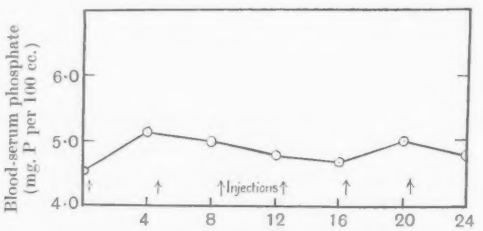

Fig 6. Effect of insulin on the blood-phosphate of rabbits. Dose as in fig. 4, curve A.

Pituitrin, therefore, not only inhibits the blood-sugar lowering action of insulin, but also the calcium raising action. The effect of pituitrin on the hormone extracted from the parathyroid gland by Collip's method is at present under investigation.

The effect of injecting insulin at 4-hourly intervals on the blood phosphorus content of rabbits was also investigated in the course of these observations. It can be seen from Fig. 6 that there was no change in the organic phosphorus content of the blood, thus differing from the effect of insulin when studied over short periods. It must also be borne in mind that these animals were fed throughout the experiments.

Discussion.

It would seem that the specificity of the parathyroid hormone remains to be proved, since an extract made from pancreas by Collip and Clark's method gave marked rises in the serum calcium content. Insulin, and to a less extent pituitrin, also raised the serum calcium content very considerably. The question naturally arises as to whether these extracts made from other tissues contain the same calcium elevating substance as do the parathyroid glands, or whether their pharmacological activity is due to an entirely different substance. The experiments detailed in this communication do not give a decisive answer to this question, but it would be reasonable to assume that the calcium raising factor is the same in all cases. Thus neither the extract made by the Collip and Clark process from parathyroid glands, nor that prepared by the same method from pancreas had any action upon the blood-sugar content of the animals. As already pointed out, little can be deduced from the inactivation experiments described earlier. The similarity in the properties of the substances extracted from the parathyroid and other tissues, such as the solubilities of the picrates, etc., suggest that the active substances are either the same or very closely related. If further experiments support this view, then the parathyroid hormone will occupy a position very similar to that of insulin. For instance, it has been shown by various workers that insulin can be recovered from tissues other than the pancreas. Large yields were obtained from salivary glands, kidneys and spleen [Baker, Dickens and Dodds, 1924]. Considerable quantities were also extracted from the tissues of a person who had died in diabetic coma. This observation corresponds to that of Best, Smith and Scott [1924], who showed that the muscles of a depancreatised dog contained large quantities of insulin. A calcium elevating substance can be extracted from the pancreas and from the pituitary body, but up to the present no observations have been made on the tissues of thyro-parathyroidectomised animals, nor has the potency of these extracts been tested upon such animals. The chemical and pharmacological similarity between the two hormones is very striking, and it is hoped that further observations upon the lines indicated will emphasise their relationship.

SUMMARY.

1. The serum calcium content of rabbits was found to vary within about 1 mg. per 100 cc. throughout the 24 hours.

2. When injected at four-hourly intervals with large doses of a parathyroid preparation made by Collip and Clark's process the serum calcium content of rabbits' blood rose during the period of the experiment.

3. The hormone yields a picrate which is insoluble in water, but is soluble in 70 % acetone, and which can be converted into a hydrochloride by Dudley's method.

4. Active material can be extracted from the parathyroid glands by means of the acetone-picric acid process.

5. Insulin, when administered in the same manner to rabbits as the parathyroid extract, was found to cause an increase in the serum calcium content. Pituitrin also gave a smaller rise, but when these two substances were administered simultaneously, both the calcium elevating powers and the blood-sugar lowering activity of insulin were inhibited. The significance of these observations is discussed.

It is a pleasure to record our thanks to Messrs Parke, Davis and Company for kindly supplying us with a quantity of frozen parathyroid glands.

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XCIII. STUDIES ON XANTHINE OXIDASE.

VII. THE SPECIFICITY OF THE SYSTEM.

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The study of the degree of specificity which an enzyme exhibits towards its substrate is always important for the proper understanding of the mechanism by which it acts. A number of systematic studies have been made on the specificity of certain hydrolysing enzymes, but up to the present no systematic study of any one oxidation-reduction enzyme appears to have been made from this point of view. This is possibly on account of the difficulty of separating these enzymes from one another. The xanthine oxidase however forms a very suitable example to choose for such an investigation, for, as Dixon and Thurlow [1924, 1] have shown, it can be prepared free from other enzymes from milk. The present paper describes a preliminary study of the specificity of this enzyme.

There is a fundamental difference to be considered between hydrolysing and oxidising enzyme systems. In hydrolysing systems we have, in addition to the catalyst, only one reactant to consider, namely, the substance hydrolysed (neglecting of course the water molecules which take part in the reaction). In this case we have only to consider the specificity of the enzyme catalyst towards this substrate. In the case of oxidising systems, however, we have, besides the catalyst, *two* reactants to consider. Before any reaction can occur in such systems there must always be present, in addition to the enzyme, both an oxidisable (reducing) substance and a reducible substance (or oxidising agent). In other words, we must have present simultaneously a hydrogen donator (hypoxanthine, etc.) and a hydrogen acceptor (oxygen, methylene blue, etc.).

The investigation of the specificity of an oxidation-reduction enzyme therefore falls naturally into two distinct sections. We have to determine how far the enzyme is specific on the one hand towards the hydrogen donator and on the other hand towards the hydrogen acceptor. In the case of the xanthine oxidase, therefore, we have first to determine how far other substances can replace hypoxanthine, using throughout a typical hydrogen acceptor such as methylene blue; and secondly we have to determine how far methylene blue can be replaced by other hydrogen acceptors, using throughout an efficient hydrogen donator such as hypoxanthine.

In the work which follows the xanthine oxidase used was prepared from milk partly by the method of Dixon and Thurlow [1924, 1], and partly by a later method which gives an extremely active preparation. It is hoped to publish details of this method shortly. Most of the experiments in which negative results were obtained were also repeated with whole milk, and the same results obtained.

The Hydrogen Donator.

In the experiments described in this section methylene blue was used throughout as the hydrogen acceptor, and various substances were tested to see whether they were capable of acting as hydrogen donators in presence of the oxidase.

Hypoxanthine and xanthine. The fact that in presence of the milk enzyme these purines rapidly reduce methylene blue was shown by Morgan, Stewart and Hopkins [1922], and the reaction was further studied by Dixon and Thurlow [1924, 2]. There can be no doubt that the same enzyme is responsible for the oxidation of both purines.

Aldehydes. The oxidation of aldehydes by methylene blue in presence of milk was shown by Schardinger [1902]. Schardinger used formaldehyde, but it appears that most aldehydes act in the same way. It is noteworthy, however, that glucose, lactose, etc., show no trace of reduction of methylene blue, even after many days with the most active enzyme preparation yet obtained.

The question as to whether the oxidation of aldehydes is brought about by the xanthine oxidase, or by a special aldehyde oxidase present together with this in the preparations used, is a difficult one. The problem has been discussed by Morgan, Stewart and Hopkins [1922] and by Dixon and Thurlow [1924, 2]. On the one hand, it seems unlikely that an enzyme so specific in other respects, as will be seen, should act upon such widely different substances as purine bases and aldehydes. On the other hand, a considerable amount of evidence was obtained by Dixon and Thurlow in support of the view that only one enzyme is concerned. I shall return to this point later.

Adenine. It was shown by Morgan, Stewart and Hopkins that adenine was able to reduce methylene blue very slowly in presence of milk. They believed that this was due to a gradual deamination of the adenine by traces of adenase with the production of hypoxanthine, which was then oxidised by the xanthine oxidase. Dixon and Thurlow [1924, 2], on the other hand, obtained some evidence that a direct oxidation of the adenine by the oxidase occurred. It cannot yet be stated definitely which view is correct.

It may be stated at once that the above-mentioned substances are the only ones I have found to be capable of acting as substrates for the xanthine oxidase.

It is, of course, necessary to use in all experiments a milk or enzyme preparation free from bacteria, since the bacteria themselves reduce methylene blue. For this reason one must always make sure by a control experiment that

the milk or oxidase solution does not reduce methylene blue by itself without the addition of a hydrogen donator. A failure to do this is apparently responsible for statements in the literature that many substances can act in a similar way to hypoxanthine in the milk oxidase system. Abelous and Aloy [1918, see also 1917], and Aloy and Valdiguie [1921] state that they found the following substances to reduce methylene blue in presence of milk or a preparation of the milk oxidase: all the aldehydes of the aliphatic and aromatic series; a large number of ptomaines; substances of the uric acid group—guanine, hypoxanthine[1], xanthine, alloxan; alkaloids—quinoline, morphine, cicutine, muscarine; toxalbumins—ricin, abrin; products of protein hydrolysis—proteoses, peptones, tryptophan; and various other substances belonging to the groups of ketones and amines (*e.g.* benzylamine). Unfortunately no experimental data were published, and it does not appear that control experiments were carried out. Since Abelous and Aloy's statement seemed to convey a totally wrong impression of the specificity of the milk oxidase, I have repeated most of their experiments, using very fresh milk and enzyme preparations free from bacteria; and, except in the cases previously mentioned and in the case of commercial peptone, which will be dealt with below, I have obtained completely negative results.

My experiments were carried out as follows. The substances were made up in 1 % aqueous solution, and all the solutions were carefully adjusted to p_H 7·6 before use. 0·5 cc. of the solution was taken, together with 5 cc. of oxidase solution in p_H 7·6 phosphate buffer and 0·5 cc. of 1/5000 methylene blue. The experiments were carried out anaerobically in vacuum tubes as usual. If in any experiment no reduction had occurred even after many hours, the tube was opened and a small amount of hypoxanthine added in order to make sure that the enzyme had not been destroyed by the substance under investigation. In all cases a rapid reduction was then obtained, showing that none of the substances tried was toxic to the enzyme. The amount of substance taken was always considerably in excess of the amount of methylene blue used.

Guanine. This was prepared in the usual way from nucleic acid, and was recrystallised several times as the hydrochloride. No trace of reduction could be observed after many hours. (The reduction-time of the enzyme solution used was about two minutes with hypoxanthine.)

Alloxan. Three specimens were used. One was prepared by the oxidation

[1] A question of priority arises here. It has been thought that Morgan, Stewart and Hopkins [1922] were the first to detect the xanthine oxidase in milk, but it will be seen that Abelous and Aloy published their results four years earlier. Abelous and Aloy, however, merely include hypoxanthine and xanthine, together with aldehydes, in a list among a large number of substances which in actual fact do *not* reduce, as will be shown below. The reduction obtained with these other substances can apparently only have been due to the presence of bacteria, and this fact invalidates their experiments with the purine bases also. Priority therefore rests with Morgan, Stewart and Hopkins.

I am asked by Sir F. G. Hopkins to state that the observations of Morgan, Stewart and Hopkins were made quite independently of those of Abelous and Aloy.

of uric acid with bromine, another by the oxidation of uric acid with nitric acid, and the third by the oxidation of dialuric acid. The last two were twice recrystallised from water. No reduction could be observed with any preparation, even after two days. The presence of alloxan was found to slow down the reduction of methylene blue by hypoxanthine to some extent. This inhibition is probably due to the same cause as the inhibition by purines observed by Dixon and Thurlow [1924, 2].

Quinoline. This was dissolved in dilute HCl, a small amount of phosphate added as a buffer, and the solution brought to neutrality with dilute NaOH. By this means a clear solution was obtained. In presence of the oxidase no trace of reduction of methylene blue could be observed.

Morphine. This was a very pure preparation of the hydrochloride. Again no action occurred.

Ricin. This was a preparation "nach M. Jakoby" obtained from the "Vereinigte Chemische Werke Aktiengesellschaft, Charlottenberg." It was dissolved in phosphate buffer. Again the results of tests with the oxidase were entirely negative.

Tryptophan. This was a pure crystalline specimen prepared in this laboratory. Not the slightest reduction was obtained.

Ketones. Abelous and Aloy do not mention the nature of the ketones they used. Acetone certainly is not oxidised by the enzyme.

Benzylamine. A specimen of the hydrochloride obtained from British Drug Houses was used. Here again there was no action whatever.

Peptone. The case of peptone is especially important in view of certain statements in the literature. Abelous and Aloy, as stated above, include peptones in the list of substances capable of replacing hypoxanthine in the xanthine oxidase system. Aloy and Valdiguie [1921], however, state that, although commercial peptones reduce methylene blue in presence of the enzyme, the reducing substance can be extracted from the peptone by alcohol, and that after this extraction the peptone is left inactive. It is clear then that the effect is due to some substance present in the commercial peptone, and that the peptone itself can *not* replace hypoxanthine in the system. I have confirmed the observations of Aloy and Valdiguie, using both Savory and Moore's and Witte's peptone. I find however that, while the substance is extracted by ordinary 95 % alcohol, it is not extracted by absolute alcohol, or by ether.

Having regard to the method of preparation of commercial peptones, it seemed very probable that they would contain small amounts of hypoxanthine. To determine whether the activity of the commercial peptone was due to this the following procedure was adopted.

200 g. of Savory and Moore's peptone were extracted in a Soxhlet apparatus with 95 % alcohol for a day. The alcoholic extract was carefully evaporated to dryness on the water-bath, leaving a small quantity of light-brown residue. This was extracted with ether, and the residue was dissolved in water. A

few drops of this solution reduced methylene blue actively in presence of xanthine oxidase. To this solution was then applied the ordinary method for the preparation of pure hypoxanthine from meat extract, as follows. The solution was made alkaline with ammonia, and ammoniacal silver nitrate was added: the resulting flocculent precipitate was filtered off and washed with dilute ammonia. The filtrate, after acidification, removal of the excess silver as chloride, and neutralisation, was found to be inactive. The precipitate was suspended in nitric acid of specific gravity 1·1, and dissolved by warming. On cooling a white crystalline precipitate separated out, resembling that usually obtained with hypoxanthine. This was filtered off and recrystallised from fresh nitric acid (sp. gr. 1·1). The pure white precipitate was then filtered off, suspended in a few cc. of water, and decomposed with H_2S. The silver sulphide was filtered off, the H_2S removed by aeration and finally by thorough shaking *in vacuo*, and the solution neutralised with dilute soda. A very small quantity of this solution was sufficient to reduce methylene blue in presence of xanthine oxidase, while control experiments without the oxidase showed that this reduction was not due to traces of H_2S remaining in the solution.

I think these facts may be said to show that the reducing properties of commercial peptones in presence of the xanthine oxidase are due to the traces of hypoxanthine they contain.

Aloy and Valdiguie give the following experiment as additional evidence that protein hydrolysis products can be oxidised by the milk oxidase. Fresh and boiled milk were digested separately with pancreatin at 37° for 12 hours. Both were then boiled and filtered, and portions of both filtrates were added to fresh milk and methylene blue. The methylene blue was reduced in the case of the milk which had been boiled before digestion, but no reduction occurred in the case where the milk was not boiled before digestion. They explain this result as follows. During digestion, oxidisable hydrolysis products are produced from the milk proteins. In the case of the fresh milk these are oxidised as soon as formed by the oxidase present in the milk and atmospheric oxygen. In the other case the initial boiling has destroyed the oxidase, and therefore the hydrolysis products do not become oxidised, and remain in solution. Now on adding fresh milk oxidase and methylene blue to both solutions one would naturally obtain a reduction in the latter case only.

An alternative explanation however is that the pancreatin itself contains hypoxanthine (as is extremely probable, considering its nature). When this is added to fresh milk the hypoxanthine will be rapidly oxidised by the oxidase, and hence there will be no reduction when the solution is ultimately tested with fresh milk and methylene blue. In the other case no xanthine oxidase is present, and thus the hypoxanthine will remain, and will account for the reduction finally observed. The digestion probably has nothing to do with the effects observed. I have tested two different samples of pancreatin with xanthine oxidase and methylene blue and found them both to reduce rapidly, although no reduction occurred in the absence of the xanthine oxidase. This

makes it very probable that the effects are in reality due to hypoxanthine and not to protein hydrolysis products.

The observations of Bach [1914] are probably to be explained in a similar manner. Bach found that "erepton," a preparation of very completely digested protein, was able to replace aldehyde in the milk oxidase system; and he explained this by supposing that the reduction was due to the actual formation of aldehydes from the amino-acids by means of the Strecker reaction. He was in fact able to obtain aldehydic substances by the distillation of erepton solutions. He admits, however, that the erepton solutions themselves contained no aldehydes, and that these were only formed during the process of distillation, so that his explanation appears to be unsatisfactory. It seems much more likely that the reduction in presence of milk was due, not to aldehydes, but to small amounts of hypoxanthine in the erepton, perhaps derived from the proteolytic enzyme preparations used in its production.

Sbarsky and Michlin [1925] state: "Als Sauerstoffakzeptoren für die Wirkung des isolierten Ferments können Aldehyde, Eiweissabbauprodukte, Xanthin und Hypoxanthin fungieren." The only evidence however adduced to show that protein breakdown products can act in this way is the fact that Liebig's extract of meat will reduce methylene blue in the presence of the xanthine oxidase. But this is to be expected, since Liebig's extract is so rich in hypoxanthine that it is the source from which this substance is usually obtained in the laboratory. (A careful reading of Sbarsky and Michlin's paper suggests, however, that it is just possible that by the term protein breakdown products they may mean hypoxanthine and xanthine, although the statement quoted does not give this impression.)

Summarising the facts, then, we may say that there appears to be no evidence that peptones, etc., can act as substrates for the xanthine oxidase; and that in cases where they have been obtained free from hypoxanthine they do not do so.

We now turn to the consideration of a number of substances which one might think would be more likely to be oxidised than most of those dealt with above, substances which are known to be oxidised in tissues by similar systems. Thunberg [1920] and others have demonstrated the oxidation of a number of substances by methylene blue in the presence of muscle and other tissues. These oxidations are brought about by enzymes of the same type as the milk oxidase. Experiments were therefore carried out to see whether the xanthine oxidase itself might not oxidise these substances to some extent.

Formate, acetate, lactate, succinate, citrate, glutaminate. These were the more important substances found by Thunberg to be oxidised by muscle, in presence of which they reduce methylene blue with great rapidity. In presence of the xanthine oxidase, however, they all gave absolutely negative results, and not the slightest action could be observed. The resistance to oxidation of these substances by the xanthine oxidase is in marked contrast to the ease with which they are oxidised by the muscle enzymes. On the other hand, it is interesting to note that hypoxanthine and xanthine are not oxidised by

muscle, which contains only insignificant traces of the xanthine oxidase. (These traces are so small that they can only be detected by making a very concentrated extract of washed muscle in alkaline phosphate.) Thus it appears that none of the muscle enzymes can oxidise hypoxanthine, while the xanthine oxidase can oxidise none of the muscle donators tried.

A number of other substances have previously been shown by other workers not to be oxidised by the xanthine oxidase.

Caffeine, theobromine, uracil, thymine, cytosine, histidine, uric acid. Morgan, Stewart and Hopkins found that none of these substances was able to bring about any reduction of methylene blue in presence of fresh milk, and I have confirmed these results, using preparations of the xanthine oxidase.

Amino-acids. Bach [1913] has shown that none of the following substances is oxidised by milk in presence of either methylene blue or nitrate as a hydrogen acceptor: glycine, tyrosine, alanine, serine, leucine, aspartic acid. He also obtained negative results with pyruvic and oxalacetic acids.

Summarising the results of this section we may then say that of about 35 substances of biological interest which have been examined only three, namely, xanthine, hypoxanthine and aldehydes (possibly also adenine to a slight extent), are oxidised by the milk oxidase.

The Hydrogen Acceptor.

Here we find a very different state of affairs. So far from the enzyme exhibiting any specificity towards the hydrogen acceptor, it appears that the chemical nature of the acceptor is quite immaterial. Almost any reducible substance (oxidising agent) seems to be able to oxidise hypoxanthine in presence of the xanthine oxidase. In this section will be described the results of experiments with various substances as hydrogen acceptors, using hypoxanthine throughout as the hydrogen donator. Hypoxanthine was chosen because, of the three substrates of the oxidase, it is the one which gives the most rapid reduction. In all cases proper control experiments were carried out to make sure that neither the enzyme solution alone nor the hypoxanthine alone reduced the hydrogen acceptor, at any rate with a velocity sufficient to invalidate the experiment. It was found in certain cases that only a small concentration of the oxidising agent could be used, as higher concentrations destroyed the enzyme. It has already been shown by Dixon and Thurlow [1924, 1] that the xanthine oxidase is somewhat easily destroyed by oxidation.

Methylene blue. This has already been fully dealt with.

Thionine. The behaviour of thionine, which is methylene blue minus the four methyl groups, is precisely similar.

Guaiacum blue. It was frequently noticed, when testing the oxidase system for the presence of peroxide [Thurlow, 1925] with guaiacum tincture and peroxidase, that if the test-tube were allowed to stand for a short time the deep blue colour at first produced was decolorised, except for a layer at the surface of the liquid where it was exposed to the air. This was found to be

due to the reduction of the guaiacum blue by the xanthine oxidase hypoxanthine system. It is of interest in this connection to note that Mrs Callow [1926] found when testing bacteria for peroxidase that the blue colour produced in the test was similarly discharged, and she showed that this decoloration was due to those thermolabile agencies in the bacteria which reduce methylene blue.

Oxygen is of course the normal hydrogen acceptor when the reaction takes place *in vivo*. Morgan, Stewart and Hopkins [1922] studied the oxidation of both hypoxanthine and xanthine by oxygen and Wieland [1914] found that aldehyde was oxidised aerobically in presence of milk. Thurlow [1925] showed that in these reactions the oxygen becomes reduced to hydrogen peroxide, and Dixon and Thurlow [1925] showed that activation of the oxygen by iron, as assumed by Warburg [1923], is not necessary in this system.

Hydrogen peroxide. It was found by Thurlow [1925] that the hydrogen peroxide produced during the aerobic oxidation ultimately disappeared again as the reaction proceeded. This observation, taken in conjunction with the observation of Morgan, Stewart and Hopkins [see also Harrison and Thurlow, 1926] that the amount of oxygen used up was exactly equal to the amount theoretically necessary for the oxidation of the hypoxanthine to uric acid, must mean that the hydrogen peroxide is used up in the oxidation of hypoxanthine, becoming itself reduced to water. This is in harmony with the observation of Wieland [1921] that hydrogen peroxide can act as a hydrogen acceptor in catalytic dehydrogenation systems. It should be mentioned that hydrogen peroxide is unable to oxidise hypoxanthine in the absence of the xanthine oxidase as separate control experiments show. Moreover, hydrogen peroxide, except in very small concentrations, destroys the oxidase [Dixon, 1925], and under certain conditions one may get complete destruction of the oxidase while the solution still contains hypoxanthine and peroxide. In this case the peroxide does not disappear from the solution; even after many hours the strength of the peroxide test remains exactly the same. On the addition of more oxidase however the peroxide disappears.

It is interesting to note that in this reaction we have an instance of the mechanism which Wieland [1921] assumes to account for peroxidase action. This system is however certainly not a peroxidase, with which class of enzymes it has nothing in common. Peroxidases appear to act by quite a different type of mechanism.

Nitrate. It was shown by Dixon and Thurlow [1924, 3] that nitrate could oxidise hypoxanthine and xanthine in presence of the xanthine oxidase, becoming itself reduced to nitrite. It was shown that the nitrate behaved exactly like methylene blue in this system. Bach [1911] showed that aldehydes reduce nitrates in presence of milk.

Dinitrobenzene. Lipschitz [1921] found that tissues were capable of reducing aromatic nitro-compounds, such as *m*-dinitrobenzene, which he found to be reduced to *m*-nitrophenylhydroxylamine. He repeated Thunberg's [1920]

work on the enzymes of muscle, using *m*-dinitrobenzene instead of methylene blue as the hydrogen acceptor, and obtained exactly the same results as Thunberg. It appears therefore that the tissue enzymes are able to work with dinitrobenzene as well as with methylene blue. Lipschitz used two routine tests for the reduction of the dinitrobenzene; first, the development of the yellow colour of the reduction product, which could be used for the colorimetric estimation of the reduction, since the dinitrobenzene itself is colourless; and secondly, the production of a strong red colour on the addition of alkali. On testing the xanthine oxidase I obtained similar results. To 5 cc. of oxidase solution containing 0·5 mg. of hypoxanthine was added about 0·1 g. of powdered *m*-dinitrobenzene, and the evacuated tube shaken at intervals. A yellow colour was quickly produced, which steadily deepened in intensity. On the addition of NaOH the solution turned deep red, but with a distinct purple tinge which may have been due to some other reduction product.

Picric acid. Abelous and Aloy [1903] have found that picric acid is reduced to picramic acid by certain reducing systems in tissue extracts. Tests were therefore carried out to see whether the xanthine oxidase system would behave similarly.

A few drops of dilute picric acid solution were added to 5 cc. oxidase solution containing 0·5 mg. of hypoxanthine and the solution was allowed to stand anaerobically. The deep orange-brown colour characteristic of picramic acid was rapidly developed, while in a similar control experiment without hypoxanthine there was no change whatever in the colour of the solution, which remained bright yellow.

Iodine. This also was found to act as a hydrogen acceptor in the oxidase system. One drop of approximately decinormal iodine solution was added to 5 cc. of enzyme solution with 0·5 mg. of hypoxanthine and a drop of starch solution. Side by side with this a control experiment was carried out containing the iodine, starch and oxidase, but with no hypoxanthine. Almost immediately the colour in the tube with hypoxanthine began to fade, and in a few minutes the solution was decolorised. At this time the control tube was apparently coloured as deeply blue as at first, and the contrast was very striking, but after a time the control tube also became decolorised, owing to the iodine attacking the protein of the enzyme preparation. The time taken for this decoloration, however, was about five times as long as that taken in presence of hypoxanthine, so that the results were quite convincing. A control experiment with iodine and hypoxanthine alone in the absence of the enzyme gave no decoloration of the iodine. Experiments made with bromine instead of iodine were inconclusive, as the bromine was too rapidly decolorised by the proteins present.

Permanganate. The results here were also quite definite. The enzyme was found to be easily destroyed by potassium permanganate, but by using only enough of the latter to give a quite definite pink colour to the solution this difficulty could be overcome. The experiments were carried out in the same

way as those with iodine. After a few minutes the pink colour in the tube with enzyme and hypoxanthine had completely disappeared, and a brownish cloud, presumably of manganese dioxide, had appeared, while the control tube containing enzyme only was still quite pink. The same changes were also observed in the control tube, but only after a period about three times as long as the decoloration-time with hypoxanthine. But by the time the control tube had become decolorised further changes had taken place in the tube containing hypoxanthine. The brownish cloud of manganese dioxide had apparently become further reduced by the system, and had redissolved, leaving the solution quite clear and colourless. A control experiment with hypoxanthine and permanganate in the absence of the oxidase showed that the permanganate was not reduced by the hypoxanthine alone.

Alloxan[1]. It has been shown earlier in this paper that alloxan will not act as a hydrogen donator with the xanthine oxidase. It appeared possible, however, that it might act as a hydrogen acceptor in the oxidation of hypoxanthine by the xanthine oxidase, becoming itself reduced to dialuric acid. Now Bijlmann [1923] has shown that the alloxan-dialuric acid system is one which gives a definite oxidation-reduction potential, so that the reduction of alloxan can be investigated by the electrometric method. The experiment was carried out as follows. 5 cc. of a 1 % solution of the active enzyme preparation in p_H 7·6 phosphate buffer together with 1 cc. $M/100$ alloxan solution were placed in a reaction vessel of about 25 cc. capacity, and a stream of nitrogen was bubbled through in order to keep the solution stirred and to remove oxygen. The reduction potential was determined by means of a gold electrode immersed in the solution, connection being made with a standard calomel cell by means of an agar salt-bridge. The potential readings were accurate to 1 mv. A preliminary experiment showed that the potential given by alloxan was perfectly steady and not affected by the presence of the enzyme. The potential (relative to the normal hydrogen electrode) was initially + 288 mv., and after standing for a considerable time the reading was exactly (within 1 mv.) the same. Hypoxanthine solution containing 0·4 mg. was then added from a burette, when the following readings were obtained:

Time after addition of hypoxanthine (minutes)	E_h (mv.)	Time after addition of hypoxanthine (minutes)	E_h (mv.)
0	+288	5	+160
½	270	6	146
1	250	8	130
2	217	10	117
3	195	15	98
4	175		

The reduction of the alloxan is shown by the rapid change in the potential reading in the direction of higher reduction potential. To judge from some not very accurate measurements on the potential of alloxantin at this p_H, the last reading given corresponds to about 60 % reduction of the alloxan. It

[1] This and the following experiment were carried out jointly with Dr Keizo Kodama.

will be seen that only enough hypoxanthine was added to reduce about three-quarters of the alloxan, so that, as the reaction was still proceeding, although rather slowly, at the end of the experiment, the results are in approximate agreement with theory.

I have repeatedly found in control experiments that the oxidase plus hypoxanthine alone gives no reduction potential. A drift of a few (10–20) millivolts in either direction may be observed on the addition of the hypoxanthine, but there is no definite potential change either with hypoxanthine or aldehyde, or with fresh milk in place of the oxidase preparation.

Quinone. The quinone-quinol system also gives a definite oxidation-reduction potential, and the reduction of quinone can therefore be followed electrometrically in a similar manner. On testing 5 cc. of the enzyme solution with 1 cc. of $M/100$ quinone a slight drift of about 10 mv. in the potential reading occurred, but after a few minutes the potential had become steady; and after waiting a further period to make sure of this, 1 mg. of hypoxanthine was added. The readings were as follows:

Time after addition (minutes)	E_h (mv.)	Time after addition (minutes)	E_h (mv.)
0	+312	28	+87
4	286	29	65
8	278	30	50
12	272	35	32
20	255	60	32
26	224		

The reduction of the quinone is shown by the change in potential as before. The peculiar form of the reaction curve however, which has been observed in every experiment, requires further investigation.

Clark's "Reduction-potential Indicators." W. Mansfield Clark [1923–25] has recently introduced a series of reducible dyestuffs, belonging to the indophenol and indigo groups, whose oxidation-reduction potentials have been accurately measured; and he suggests their use as indicators for the determination of r_H[1], just as the more familiar indicators are used to determine p_H. It was thought that by using these dyes as hydrogen acceptors in the xanthine oxidase system it might be possible to estimate the reducing power of this system. So far as I am aware this method has not been previously applied to any oxidation-reduction enzyme system.

The technique used was exactly the same as with methylene blue. The dyes[2] were made up in aqueous solutions equivalent in strength to the 1/5000

[1] The r_H scale introduced by Clark is a method of expressing the reducing power of a solution, strictly analogous to the p_H scale of acidity. For an explanation of the r_H notation reference must be made to Clark's papers, but the following brief remarks will not be out of place. By definition $r_H = -\log P$, where P is the pressure of hydrogen gas in a platinum surface in equilibrium with the solution. Thus a solution with a high reducing power has a small r_H value, and *vice versa*. In other words a substance with a low r_H is itself more difficult to reduce than one with a high r_H. At $r_H = 0$ the solution will evolve hydrogen gas spontaneously at the platinum surface; at $r_H = 41$ oxygen gas will be given off. The r_H is calculated from potential measurements.

[2] For these dyes I am much indebted to Dr and Mrs Needham. I understand that they were sent to Dr Needham by Professor Mansfield Clark.

methylene blue used previously, and the experiments were all carried out anaerobically at 14°. A 2 % solution of xanthine oxidase preparation in phosphate buffer solution was used, and the quantities were as follows: 1 cc. oxidase solution + 1 cc. p_H 7·6 phosphate buffer + 0·5 cc. of the dyestuff solution + 0·2 mg. hypoxanthine. The results of the experiments are given in the following table:

Indicator no.	Time for complete reduction	Approximate r_H range	No. of sulphonate groups
1	13 m.	7·2–11·2	2
2	50 m.	8·3–12·3	3
3	8 hrs.	10 –14	4
3*a*	1 m. 35 s. 1 „ 30 „	13 –17	0
4	5 „ 30 „	15·2–19·2	1
5	2 „ 20 „	15·4–19·4	1
6	1 „ 30 „	17·3–21·3	0
7	1 „ 10 „	17·7–21·7	0
8	1 „ 15 „	18·3–22·3	0
9	55 „	19 –23	0

For convenience the indicators are referred to by numbers. Their names are as follows:

No.

1. Indigotine disulphonate.
2. Indigotine trisulphonate.
3. Indigotine tetrasulphonate.
3*a*. Methylene blue.
4. 1-Naphthol-2-sulphonate-2:6-dichloro-indophenol.
5. 1-Naphthol-2-sulphonate-indophenol.
6. *o*-Cresol-2:6-dichloroindophenol.
7. *o*-Cresol-indophenol.
8. 2:6-Dibromophenolindophenol.
9. *o*-Chlorophenolindophenol.

The dyes are given in order of their reduction-potentials, no. 1 requiring the greatest energy to reduce it, and no. 9 being the easiest to reduce. The r_H range is given from 1 % to 99 % reduction, but of course the *useful* range is more limited.

The first important point which appears from these results is that the xanthine oxidase system completely reduces all the indicators; that is to say, its reducing power is so great that it lies beyond the region at present covered by indicators. Its r_H must be below 7.

The second point is the effect of sulphonic groups on the velocity of reduction of the dyes. It will be seen from the table that indicators 3*a*, 6, 7, 8 and 9, which are not sulphonated, are all reduced rapidly at about the same rate. On the other hand, 4 and 5, which contain one sulphonic group, are definitely slower; while 1, 2 and 3, which contain 2, 3 and 4 sulphonic groups respectively, show the very large effect on the reduction rate of more than one such group. It is to be noted that among the last three cases mentioned, no. 3, the one which, from reduction potential data, we know is the easiest of the three to reduce, has the slowest reduction velocity; while no. 1, the most difficult to reduce, is reduced quickest. This clearly shows that the effect is simply due to the sulphonic group. Since this group is strongly dissociated, each group will give one negative charge to the molecule, so that the effect may be one of charge merely.

The point is of importance in connection with attempts to determine the r_H of biological systems which are not in equilibrium by means of these indicators. For instance, J. and D. M. Needham [1925, 1926, 1, 2] have made micro-injections of these dyes into a large number of different cells in an investigation of the internal r_H of the living cell. They find that in the majority of the cells studied, 6, 7, 8 and 9 were reduced, while 1, 2, 3, 4 and 5 remained in the oxidised form. (Methylene blue was not used in most cases.) They point out that the change from reduced to oxidised does not correspond with the change from the indophenol to the indigo series, and they conclude that the change is not due to a change in the chemical structure of the dye. An examination of the list of dyes shows however that dyes 1–5 are all sulphonated, while 6–9 contain no sulphonic groups; so that it may be said that sulphonated dyes exist in these cells in the oxidised form, and non-sulphonated dyes in the reduced form. Now a dye which is only slowly reduced in a cell will be re-oxidised, as fast as it is reduced, by oxygen if this is present, or possibly by other hydrogen acceptors in the cell. In this case the dye will be present mainly in the oxidised form. On the other hand, if the dye is reduced more rapidly than it can be oxidised, it will be mainly in the reduced form. This would be equally true no matter whether the reduced or oxidised form of the dye were used initially.

The reducing power of the living cell is due, in large measure at any rate, to systems of the same type as the xanthine oxidase. A consideration of these facts then suggests that the apparent results obtained may depend not so much upon the r_H of the cell as upon whether the indicator used is sulphonated or non-sulphonated.

In this connection it is significant that the Needhams [1926, 2] found one cell, *Nyctotherus cordiformis*, which when deprived of oxygen reduced nos. 3 and upwards. Now in presence of oxygen this cell reduced only no. 6, and was unable to reduce 4 and 5. In other words, the apparent r_H of the cell rose just to the junction of the sulphonated with the non-sulphonated series. This result would seem to be due to an effect of the kind just pictured.

A further, somewhat different, criticism applies to the case where the oxidised form only is added initially. Unless the time of observation is very long the more slowly reacting indicators may not have reached their final stage by the end of the observation. For instance, if we were determining the r_H of the xanthine oxidase system with indicators 3 and 3*a*, and the observation only lasted a few minutes, we should say that as 3*a* was completely reduced the r_H must be about 13 or less, while as 3 remained completely oxidised the r_H must be above 14. Needham's observations generally lasted only a few minutes.

Methylene blue forms a very interesting case. As will be seen from the table, it is a non-sulphonated dye falling within the r_H range of the sulphonated dyes. If what has just been said is correct, we might possibly expect, in a biological system with a small r_H value, to find methylene blue (3*a*) reduced,

while 4 and 5 are oxidised, 6 again being reduced. It is a significant fact that J. and D. M. Needham [1925, p. 276] found that methylene blue was rapidly reduced[1] by the amoeba, while 4 was oxidised, 5 was partially reduced, and 6–9 completely reduced. 1–3 were not reduced [1926, 2]. Needham [1926, 1] also found that only the non-sulphonated dyes were capable of staining *Paracentrotus* egg cells.

Glutathione. The oxidised or disulphide form of glutathione was shown by Hopkins [1921] to act as a hydrogen acceptor for certain tissue systems, whose nature has up to the present not been determined. Muscle tissue, for instance, rapidly reduces the disulphide to the sulphydryl form, but it was found by Hopkins and Dixon [1922] that none of the better-known oxidation-reduction enzymes of muscle was able to effect this reduction. On testing the xanthine oxidase system it was found that this also was unable to reduce glutathione. Xanthine oxidase, hypoxanthine and oxidised glutathione solutions were placed in a vacuum tube and the mixture allowed to stand anaerobically for many hours, after which the nitroprusside test was applied to the solution. The test was absolutely negative. It appears from other considerations that the disulphide compound can only be reduced by systems having a very high reducing power, and it seems that the xanthine oxidase system has not a sufficiently low r_H to effect this reduction.

Discussion.

The results given in the preceding sections bring out clearly the main point of this work, namely, that the xanthine oxidase exhibits a remarkable specificity as regards the hydrogen donator (the substance oxidised), but none at all as regards the hydrogen acceptor (oxidising agent). The system would be a perfect example of clean-cut specificity were it not for the fact that aldehyde can act as the hydrogen donator in addition to the purine bases. Neglecting aldehyde for the moment, however, we can say that the enzyme is in the highest degree specific towards the hydrogen donator. In the purine series itself this is very marked: hypoxanthine and xanthine appear to be the only substances oxidised, and the introduction of amino or methyl groups into the molecule is sufficient entirely to prevent the enzyme acting on it. It is hoped to carry out a further and more detailed investigation of the purine substances from this point of view. Again, outside the purine series, the oxidase was found to be without action on any of the considerable number of substances tested, although many of these are known to be readily oxidised by other enzymes of a similar type to the xanthine oxidase.

It should be pointed out here, in connection with these negative experiments, that the technique used was capable of detecting extremely slow reactions. With the solutions used, the time for complete reduction of the methylene blue by hypoxanthine was about two minutes. As the tubes were

[1] Too much stress must not be laid on this, as they were later unable to repeat this result [1926, 2], but it appears that Clark has also observed this anomaly with methylene blue [Needham, 1926, 2, p. 389].

allowed to stand for 6–8 hours in most cases, and in some cases for much longer than this, and as a reduction of one-fifth of the dye is observed with ease, it follows that if these substances are oxidised at all they are oxidised at a velocity less than one two-thousandth of that of hypoxanthine.

With regard to the case of aldehyde, it cannot be maintained that it is definitely established that the oxidations of aldehyde and purines are due to the same enzyme. If they are not, then *both* enzymes must be remarkably specific. On the other hand, there appears to be a fairly strong balance of evidence in favour of identity, although a certain part of this can be explained away by supposing that we have two different enzymes adsorbed on the same colloid.

There appears, however, to be no need to suppose that a specific catalyst is necessary for the activation of aldehyde. Bredig and Sommer [1909] have shown that aldehydes are activated even by a non-specific catalyst like colloidal platinum, a catalyst which I have found to be quite unable to activate hypoxanthine. I found a platinum sol which rapidly reduced methylene blue in presence of aldehyde to show no reduction whatever when hypoxanthine was substituted for aldehyde. Thus it appears that hypoxanthine requires a specialised catalyst for its activation, while aldehydes are easily activated and do not necessarily require a special catalyst. It is quite certain that there are other enzymes which will oxidise aldehydes, but which are altogether without action on hypoxanthine. Aldehydes are also activated by amino-acids, as well as by a number of inorganic catalysts. It must be remembered that aldehydes are very easily oxidisable substances, even in the absence of catalysts, and in this respect they are quite unlike substances such as hypoxanthine and succinic acid which are extremely difficult to oxidise in the absence of their respective enzymes.

With regard to the hydrogen acceptor, it is quite clear that no specificity at all can be observed. It is only necessary to mention a few examples, *e.g.* methylene blue, oxygen, nitrates, permanganate, iodine and alloxan, to show that the exact chemical nature of the substance seems to be immaterial. One might almost say that any oxidising agent, which does not destroy the enzyme, will bring about the oxidation of hypoxanthine in presence of the xanthine oxidase.

The facts brought out in this work are in harmony with, and give further support to, the view that the oxidase activates the hydrogen donator and not the acceptor.

It is interesting to compare this state of affairs with the case of the peroxidase system, where it is the hydrogen acceptor (the peroxide) which is activated. Here we find the opposite result, and there appears to be little or no specificity towards the hydrogen donator. Guaiacum, benzidine, quinol, *p*-phenylenediamine, naphthol, nitrites, leucomalachite green, formates and many other substances are oxidised; in fact what applies to the hydrogen acceptor in the case of the xanthine oxidase applies here to the hydrogen donator.

How far the results obtained with the xanthine oxidase apply also to the other enzymes of the same type is a question about which little can be said at present. The observation of Lipschitz that dinitrobenzene gave throughout the same results as methylene blue with muscle tissue is good evidence that the hydrogen acceptor is in general unspecific. There is good evidence also that the hydrogen donator is specific, although the degree of specificity is as yet undetermined.

SUMMARY.

1. Using the methylene blue technique, out of 35 substances which have been tested, the only ones which have been found to be oxidised by the xanthine oxidase of milk are: hypoxanthine, xanthine, aldehydes, and possibly adenine to a slight extent.

2. The following can act as hydrogen acceptors in oxidising hypoxanthine in presence of the xanthine oxidase: methylene blue, thionine, guaiacum blue, all Clark's "reduction-potential indicators," oxygen, hydrogen peroxide, nitrates, dinitrobenzene, picric acid, permanganate, iodine, alloxan, quinone.

3. The oxidase shows a high degree of specificity towards the hydrogen donator, but none at all towards the hydrogen acceptor.

4. Certain facts are described which show that caution must be exercised in the interpretation of results obtained by the application of Clark's oxidation-reduction indicators to biological systems.

The writer wishes to express his sincere thanks to Sir F. G. Hopkins for his continued interest in this work and for helpful advice.

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XCIV. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

PART I. POSITION ISOMERISM IN RELATION TO THE MIOTIC ACTIVITY OF SOME SYNTHETIC URETHANES.

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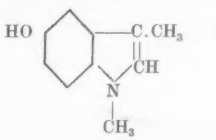
PHYSOSTIGMINE occurs in the ripe seed of *Physostigma venenosum*, Balfour. It is employed therapeutically in virtue of its power, when applied locally to the eye, of producing miosis and simultaneously causing a reduction in intra-ocular pressure. Its general pharmacological action is to stimulate the peripheral nerve-endings of the parasympathetic system, its action in this respect being antagonistic to that of atropine. The particular grouping responsible for this specific action of physostigmine has hitherto escaped recognition, partly, no doubt, owing to the incomplete knowledge which, until recently, has existed regarding the structure of this alkaloid. It has been known since the work of Salway [1912] and Straus [1913, 1914], who revised the views of earlier workers, and of Polonovsky and Nitzberg [1916], who effected the partial synthesis of physostigmine by allowing eseroline and methyl isocyanate to interact under appropriate conditions, that physostigmine is the methyl carbamido-derivative of a phenolic compound, eseroline, which is produced when the alkaloid is hydrolysed. During an investigation of the constitution of physostigmine the author [1924] succeeded in synthesising the ethyl ether of a substance, physostigmol, which had previously been obtained by Straus by the dry distillation of eseroline methiodide. Physostigmol was thus shown to have the structure I, a result which, combined with the results of other workers mentioned above, indicated that grouping II was present in the physostigmine molecule. Subsequently the work was extended by Stedman and Barger [1925] who showed that the structure of physostigmine could be



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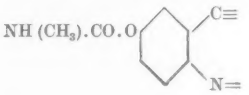
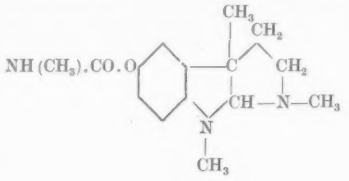


II

represented by formula III. In view of the fact that eseroline does not possess the pharmacological properties of physostigmine, the synthesis of physostigmol and the demonstration that grouping II was present in the alkaloid led to the suggestion that the physiological properties of physostigmine depended upon the fact that the alkaloid was a phenyl ester of methylcarbamic acid (*i.e.* a urethane), and experiments were at once undertaken to determine whether similar esters of simpler phenols would possess the same properties.



III

The number of phenols from which urethanes of the required type could be prepared is, of course, extremely large and it was decided, in the first instance, to confine the work to those of a simple nature, bearing in mind the necessity for the introduction of a basic group in order to permit the formation of soluble salts. The simplest phenols fulfilling this last condition are the aminophenols. In view, however, of the fact that the method contemplated for the preparation of the urethanes consisted in allowing the phenol to interact with methyl isocyanate and that the latter substance also reacts with amino-groups with the formation of substituted urea derivatives, the use of the aminophenols would lead to the formation of unnecessarily complicated molecules in which the urea grouping might conceivably mask the properties of the urethane grouping and, in any case, would cause the compounds to be of such a feebly basic nature that their salts would undergo hydrolytic dissociation with the production of strongly acid solutions and possibly the precipitation of the base. While this difficulty might be overcome by modifying the method of preparation, it appeared preferable to eliminate entirely the possibility of such a complication by methylation of the amino-group. The urethanes described in the experimental portion are therefore mainly derivatives of the three isomeric dimethylaminophenols. Variations have been made in the urethane grouping itself in order to determine what influence this had on the physiological properties. A few preliminary experiments with other phenols have also been carried out. The results of these together with a description of some compounds prepared incidentally in the course of this work are included in the experimental portion.

Experimental.

Preparation of o-, m- *and* p-*dimethylaminophenol.* Of the three isomerides, only the *meta*-compound, which is an intermediate in the preparation of dyestuffs, can be obtained commercially; this source of the phenol was utilised during the later stages of this investigation, but in the first experiments it

was prepared by a method similar to that employed in the case of the *ortho*- and *para*-isomerides and described below. *o*-Dimethylaminophenol was prepared by Griess [1880] by the distillation of the corresponding methochloride under normal pressure, and the *para*-compound by Pechmann [1899] by similar means, using, however, the quaternary iodide and carrying out the distillation under diminished pressure. The latter method was adopted in the case of all isomerides. For the preparation of the quaternary iodides it was found convenient to modify the methods of Griess and Pechmann. The dimethylaminophenol was dissolved in methyl alcohol, an equal weight of finely powdered, anhydrous sodium carbonate and slightly more than three molecules of methyl iodide added, and the mixture refluxed for about 10 hours, small additional quantities of methyl iodide being occasionally added to compensate for that carried out of the condenser by the carbon dioxide liberated in the reaction. In the case of the *meta*- and *para*-isomerides, 40 cc. of methyl alcohol were employed for 10 g. of aminophenol. When the reaction was complete the mixture was cooled and the methiodide filtered and recrystallised from methyl alcohol. In this way 10 g. of aminophenol yielded 20 g. of pure methiodide. For the preparation of the *ortho*-compound 50 cc. of methyl alcohol were used for 10 g. of aminophenol. The methiodide did not, in this case, separate from the reaction mixture but was precipitated by addition of dry ether (yield: 17 g. from 10 g. of aminophenol). It is suitable in this form for distillation, but may, if desired, be purified by recrystallisation from water.

Owing to the ease with which the dimethylaminophenols undergo oxidation with the formation of coloured products, these substances were only prepared in small quantities as required for immediate use. The quaternary iodide was distilled under diminished pressure, the distillate taken up in ether, filtered from any methiodide which had been formed by recombination, and the ether evaporated.

Urethanes derived from *p*-dimethylaminophenol.

p-*Dimethylaminophenyl ester of methylcarbamic acid.* 1·4 g. of *p*-dimethylaminophenol were dissolved in dry ether (5 cc.), an excess of methyl isocyanate was added, and the mixture left overnight. The urethane, which had meanwhile separated in crystalline form, was then filtered and washed with ether (yield 1·3 g.). When recrystallised from alcohol it forms lustrous plates which melt at 131°.

The *hydrochloride* was obtained by dissolving 0·7 g. of the base in an alcoholic solution of hydrochloric acid. It rapidly commenced to separate in crystalline form, and, after addition of ether, was filtered (yield 0·8 g.). When recrystallised from a mixture of acetone and alcohol, it forms prisms which melt at 157°.

Analysis. 0·2016 g. gave 0·1228 g. AgCl.
Cl found, 15·1 %; calc., 15·4 %.

The *methiodide* separates when a solution of the urethane in methyl iodide is allowed to stand for some hours. A further quantity is precipitated on the addition of ether. It can be recrystallised from alcohol, when it forms plates which melt at 170° with effervescence.

The *picrate* is only sparingly soluble in alcohol. When recrystallised from aqueous alcohol, it forms long prisms which melt at about 179° with decomposition.

p-*Dimethylaminophenyl ester of ethylcarbamic acid.* This substance was originally prepared without difficulty by a method similar to that described above for the lower homologue, when 1·9 g. of the urethane were obtained from 1·6 g. of the phenol. In subsequent experiments, however, addition of ethyl isocyanate to an ethereal solution of *p*-dimethylaminophenol caused the formation of a brown substance which rendered purification of the urethane difficult. Whether this was due to the presence of impurities in either the phenol or the ethyl isocyanate, or whether some other factor was responsible, is not at present known. It is probable that impurities were present in the isocyanate, for while the same difficulties were encountered in the preparation of other *N*-ethyl urethanes, this was not the case with the corresponding *N*-methyl urethanes. Although the formation of the brown impurities could not be entirely prevented, the following example indicates how the pure urethane may be obtained. The *p*-dimethylaminophenol prepared from 5 g. of the methiodide was dissolved in ethyl isocyanate. By the next day the mixture had solidified. It was ground up with a little ether, filtered, and washed with small quantities of ether (yield, 1·7 g.). The product so obtained was slightly brown and was purified by dissolving in benzene, adding light petroleum until the coloured impurities were precipitated, and decanting the clear supernatant liquid. The process was repeated on the brown residue and the solutions united and diluted with light petroleum. On standing the urethane separated in crystalline form. It can be recrystallised from benzene or alcohol and forms plates which melt at 123°.

The *hydrochloride*, prepared by treating the base with an alcoholic solution of hydrochloric acid and adding ether, crystallises from acetone in lustrous plates which melt at 77°.

Analysis. 0·2186 g., when dried *in vacuo*, lost 0·0148 g.
0·2038 g. (dried *in vacuo*) gave 0·1183 g. AgCl.
H_2O found, 7·3 %; calc. for $1H_2O$, 6·9 %.
Cl found, 14·4 %; calc., 14·5 %.

The *methiodide* may be prepared as in the case of the lower homologue. It may be recrystallised from a mixture of acetone and alcohol and melts at 160° with effervescence.

The *picrate*, when recrystallised from alcohol, sinters at 145° and melts at 158°.

p-*Dimethylaminophenyl ester of phenylcarbamic acid.* A solution of 1·5 g. of *p*-dimethylaminophenol in 15 cc. of ether was treated with 1·7 cc. of phenyl

isocyanate. After standing overnight the urethane which had separated was filtered and washed with ether (yield 1·9 g.). When recrystallised from alcohol it forms stout prisms which melt at 163°.

The *hydrochloride* is obtained by treating an alcoholic solution of the base with a solution of hydrochloric acid in alcohol and precipitating with ether. Recrystallised from a mixture of alcohol and ether, it forms tabular prisms which melt at 182° with effervescence.

Analysis. 0·2117 g. gave 0·1026 g. AgCl.
Cl found, 12·0 %; calc., 12·0 %.

The *methiodide* was prepared by treating 0·2 g. of the base with excess of methyl iodide and sufficient methyl alcohol to produce a clear solution. On standing, the methiodide separated. After the addition of ether it was filtered (yield 0·28 g.) and recrystallised from a mixture of methyl and ethyl alcohols when it formed plates which melted at 169°.

The *picrate* is sparingly soluble in ethyl alcohol. When recrystallised from this solvent it forms tabular prisms which melt at 177° with decomposition.

Attempted preparation of the p-*dimethylaminophenyl ester of carbamic acid.* It was thought that it would be possible to obtain this substance by passing an excess of phosgene gas through a solution of the aminophenol in an inert solvent, separating the chlorocarbonic ester of the phenol (which, it was anticipated, would be formed) from the other products, and treating this with dry ammonia. This method did, in fact, succeed in the case of the *meta*-isomeride, but with the *para*- and *ortho*-compounds only the diphenyl carbonates could be isolated from the products of the reactions. The conditions were varied by dropping an ethereal solution of the phenol into liquid phosgene cooled in a freezing mixture, but with no better results. Only the former experiment need, therefore, be described.

A stream of phosgene gas was passed through a solution of 3 g. of *p*-dimethylaminophenol in 30 cc. of ether for about half an hour. The sticky precipitate which at first formed slowly became granular, a process which was hastened by rubbing it with a glass rod. Some liquid phosgene was then added and the flask corked and left for 2 days. After this time the solution was filtered, the solid being stored in a desiccator while the filtrate was evaporated under diminished pressure. The residue from the filtrate, which should have contained the chlorocarbonic ester, was so small that it was not examined further. The solid which had meanwhile been stored in a desiccator was suspended in dry ether and a current of ammonia gas passed through the suspension. This liberated the base which dissolved in the ether. The ethereal solution was then filtered to remove the ammonium chloride which had been formed, and the ether evaporated. An oil which soon crystallised was thus obtained. This consisted of unchanged dimethylaminophenol together with some tetramethyl-*p*-diaminodiphenyl carbonate. The latter was isolated by crystallising the mixture from aqueous alcohol (yield 0·8 g.). When recrystallised from alcohol, *tetramethyl*-p-*diaminodiphenyl carbonate* forms flat prisms which melt at 157°.

The *hydrochloride*, when recrystallised from methyl alcohol, forms radiating aggregates which melt at 222° with effervescence.

Analysis. 0·1952 g. gave 0·1505 g. AgCl.

Cl found, 19·07 %; calc., 19·04 %.

The required chlorocarbonic ester could probably be prepared by the action of phosgene on the sodium salt of the phenol, but the author has not yet had an opportunity of testing this method.

Urethanes derived from *m*-dimethylaminophenol.

m-*Dimethylaminophenyl ester of methylcarbamic acid.* The dimethylaminophenol from 5 g. of the methiodide was dissolved in methyl isocyanate and the solution allowed to stand for some hours. Addition of light petroleum precipitated the urethane as an oil which soon crystallised. This was filtered and washed with light petroleum (yield 2·5 g.). It was recrystallised from aqueous alcohol when it formed tablets which melted at 85–86°.

The *hydrochloride* was prepared by dissolving 0·6 g. of the base in alcoholic hydrochloric acid. The salt separated after a short time (yield 0·6 g.). When recrystallised from a mixture of alcohol and acetone it forms prisms which sinter at 164° and melt at 170° with effervescence.

Analysis. 0·2089 g. gave 0·1293 g. AgCl.

Cl found, 15·3 %; calc., 15·4 %.

The *methiodide* can be recrystallised from a mixture of acetone and alcohol when it forms long prisms which melt at 165° with effervescence.

The *picrate*, when recrystallised from aqueous alcohol, forms stout rhombohedral prisms which darken at about 172° and melt at 177° with decomposition.

m-*Dimethylaminophenyl ester of ethylcarbamic acid.* 2 g. of *m*-dimethylaminophenol were treated with excess of ethyl isocyanate. The liquid immediately became brown and the phenol slowly dissolved. After standing for 2 days, light petroleum was added to the solution. This precipitated a brown oil. The colourless supernatant liquid was decanted and the residual oil treated with benzene and light petroleum in the manner described in connection with the corresponding *para*-compound. From the united solution so obtained, the urethane separated as a white crystalline solid. This was recrystallised twice from aqueous alcohol, when it formed colourless prisms which melted at 99–100°. The final yield obtained was 0·8 g. The considerable losses which occurred were due to the difficulty of separating the coloured by-products. The hydrochloride can be crystallised from alcohol and ether, but is hygroscopic.

The *methiodide* separates from a solution of the base in methyl iodide when this is allowed to stand for 2 days. When recrystallised from alcohol it forms prisms which melt at 152° with effervescence.

Analysis. 0·1179 g. gave 0·0788 g. AgI.

I found, 36·12 %; calc., 36·29 %.

The *picrate* is fairly soluble in alcohol but separates from this solvent on the addition of light petroleum in the form of long prisms which melt at 95°.

m-*Dimethylaminophenyl ester of phenylcarbamic acid.* 1·5 g. of *m*-dimethylaminophenol were dissolved in 10 cc. of benzene and treated with 1·7 cc. of phenyl isocyanate. The urethane separated on standing. It was filtered, washed with light petroleum (yield 2 g.) and recrystallised from alcohol when it formed prisms which melted at 159°.

The *hydrochloride*, when precipitated by the addition of ether to its alcoholic solution, forms clusters of radiating needles which melt at 180° with effervescence.

The *methiodide* is prepared by treating the base with excess of methyl iodide, and adding sufficient methyl alcohol to cause it to dissolve. After standing for a day the addition of ether precipitates the methiodide. When recrystallised from a mixture of acetone and alcohol it forms flat prisms which melt at 160° with effervescence.

Analysis. 0·1577 g. gave 0·0927 g. AgI.

I found, 31·8 %; calc., 31·9 %.

m-*Dimethylaminophenyl ester of carbamic acid.* A brisk stream of phosgene gas was passed through a solution of 4 g. of *m*-dimethylaminophenol in 30 cc. of ether for about 30 minutes. The sticky precipitate which formed at the commencement of this process was caused to assume a granular state by rubbing with a glass rod. Some liquid phosgene was then added to the mixture, after which the flask was corked and stored until the following day. The solution was then filtered, the solid being stored in a desiccator while the filtrate was evaporated under diminished pressure. The solid residue from the filtrate was suspended in dry ether and a stream of dry ammonia passed through the suspension for about half an hour. It was then filtered and the filtrate evaporated to dryness. This yielded a small quantity of crystalline material which was, however, too small for further investigation. The solid was then treated with water, which removed the ammonium chloride and any urea which may have been present, and crystallised from aqueous alcohol (yield 0·7 g.). This proved to be the required urethane. When recrystallised from alcohol it forms plates which melt at 137°. The solid which had meanwhile been stored in a desiccator yielded 2·5 g. of unchanged dimethylaminophenol.

The *hydrochloride* of the urethane is sparingly soluble in ethyl alcohol and can be conveniently crystallised from a mixture of ethyl and methyl alcohols, when it forms prisms which melt at 174° with effervescence.

Analysis. 0·1884 g. gave 0·1249 g. AgCl.

Cl found, 16·4 %; calc., 16·4 %.

Urethanes derived from *o*-dimethylaminophenol.

o-*Dimethylaminophenyl ester of methylcarbamic acid.* An ethereal solution of the *o*-dimethylaminophenol obtained from 5 g. of the corresponding methiodide was treated with excess of methyl isocyanate. After standing for some hours light petroleum was added, which precipitated the urethane. This was filtered and washed with light petroleum (yield 2·5 g.). When re-

crystallised from aqueous alcohol, it forms stout prisms which melt at 76°. The *hydrochloride* is an oil which will not crystallise.

The *methiodide* separates from a solution of the base in methyl iodide when allowed to stand for 2 days (0·2 g. base gave 0·35 g. methiodide). When recrystallised by addition of ether to its solution in acetone, it sinters at 78–80° and then melts with effervescence.

Analysis. 0·1161 g. gave 0·0807 g. AgI.

I found, 37·6 %; calc., 37·8 %.

The *picrate* crystallises from alcohol in the form of prisms which melt at 152° with effervescence.

o-*Dimethylaminophenyl ester of ethylcarbamic acid.* The *o*-dimethylaminophenol obtained from 5 g. of the corresponding methiodide was dissolved in ethyl isocyanate and allowed to stand for a day. Addition of light petroleum then precipitated a yellow oil. The yellow impurity was removed by treating the oil with benzene and light petroleum in the manner described above in connection with the isomeric *N*-ethyl urethanes. In this way the urethane was obtained in the form of colourless crystals. It was recrystallised by addition of light petroleum to its solution in benzene, when it separated slowly in the form of large prisms which melted at 61°. The *hydrochloride*, as in the case of other urethanes derived from *o*-dimethylaminophenol, could not be crystallised.

The *methiodide* separates as an oil from a solution of the base in methyl iodide. When the oil is washed with ether and allowed to stand it slowly solidifies (0·2 g. base gave 0·29 g. methiodide). It can be crystallised by slow addition of ether to its alcoholic solution. It melts at 119° with effervescence.

Analysis. 0·1206 g. gave 0·0805 g. AgI.

I found, 36·1 %; calc., 36·3 %.

The *picrate* crystallises from alcohol in the form of prisms which melt at 124°.

o-*Dimethylaminophenyl ester of phenylcarbamic acid.* 1·6 g. of *o*-dimethylaminophenol were dissolved in 10 cc. of benzene and 1·7 cc. of phenyl isocyanate added. The urethane crystallised from the solution on standing (yield 2·3 g.). When recrystallised from alcohol it forms long prisms which melt at 144°.

Attempted preparation of the o-*dimethylaminophenyl ester of carbamic acid.* A solution of 3 g. of *o*-dimethylaminophenol in 30 cc. of ether was treated with phosgene in the manner described for the corresponding experiments with the *para*- and *meta*-isomerides, and the product worked up in the same way. The ethereal solution left only negligible amounts of crystalline products. The solid, on the other hand, yielded a mixture of unchanged phenol and tetramethyl-*o*-diaminodiphenyl carbonate, from which the latter was isolated by crystallisation from aqueous alcohol (yield 1 g.). When recrystallised from aqueous alcohol *tetramethyl-o-diaminodiphenyl carbonate* forms prisms with pyramid ends which melt at 72°. The *hydrochloride* could not be obtained in crystalline form.

The *methiodide* crystallises from a solution of the base in methyl iodide when this is allowed to stand for a week (0·2 g. base yielded 0·27 g. methiodide). When recrystallised from alcohol and ether, it forms flat prisms which melt at 183° with effervescence.

Analysis. 0·1015 g. gave 0·0800 g. AgI.
I found, 42·6 %; calc., 43·5 %.

Urethanes derived from hordenine.

Methylcarbamido-derivative of hordenine. Owing to the very limited amount of hordenine available it was not possible to repeat the following experiment, which, although resulting in the preparation of the hydrochloride of the urethane in the pure state, was unsatisfactory in that the urethane itself was not isolated in a pure condition.

1 g. of hordenine was dissolved in 50 cc. of ether, treated with 1 cc. of methyl isocyanate and left for 3 days. Experiments with a test portion then showed that the hordenine was largely unchanged. The solvent was therefore evaporated and the residual oil dissolved in methyl isocyanate. After standing for a day the excess methyl isocyanate was evaporated under diminished pressure, the residual oil dissolved in benzene, and the urethane precipitated by addition of light petroleum. The oily precipitate quickly solidified and was then filtered. Unsuccessful attempts were made to crystallise it from various solvents, during which process the substance became brown in colour. It was therefore dissolved in ether and treated with alcoholic hydrochloric acid. The precipitate which formed was filtered and recrystallised from a mixture of alcohol and acetone. When obtained in this way, the *hydrochloride* of the *methylcarbamido-derivative* of hordenine forms lustrous plates which melt at 161°.

Analysis. 0·2195 g. gave 0·1218 g. AgCl.
Cl found, 13·73 %; calc., 13·73 %.

A small amount of what was probably the free base was obtained from the benzene light petroleum filtrate in the form of clusters of needles which melted at about 65°.

Phenylcarbamido-derivative of hordenine. 0·5 g. of hordenine was dissolved in phenyl isocyanate. After a short time solid began to separate. Light petroleum was then added, which caused a further precipitate to form, and the mixture filtered. The solid was crystallised by dissolving it in about 10 cc. of benzene and adding light petroleum. When crystallised in this way the phenylcarbamido-derivative of hordenine forms long prisms which melt at 119°.

The *hydrochloride* can be recrystallised from alcohol, when it forms plates which melt at 194° with effervescence.

Analysis. 0·1801 g. gave 0·0804 g. AgCl.
Cl found, 11·04 %; calc., 11·08 %.

Urethanes derived from 8-hydroxyquinoline.

Methylcarbamido-derivative of 8-hydroxyquinoline. 1 g. of 8-hydroxyquinoline was treated with a solution of methyl isocyanate in benzene. It slowly dissolved, and, on standing, the urethane separated from the solution. Light petroleum was added to complete the precipitation, when the solid was filtered and washed with dry ether (yield 1·35 g.). When recrystallised from alcohol the methylcarbamido-derivative of 8-hydroxyquinoline forms rectangular tablets which sinter at 152° and melt at 161°.

The *hydrochloride* quickly separated when 0·7 g. of the base was dissolved in alcoholic hydrochloric acid (yield 0·8 g.). When recrystallised from a mixture of alcohol and acetone it forms prisms which melt at 168° with effervescence.

Analysis. 0·1777 g. gave 0·1050 g. AgCl.
Cl found, 14·62 %; calc., 14·88 %.

Ethylcarbamido-derivative of 8-hydroxyquinoline. 1 g. of 8-hydroxyquinoline was dissolved in ethyl isocyanate. The urethane quickly separated in crystalline form. The excess of ethyl isocyanate was then evaporated under diminished pressure, light petroleum added to the residue and the suspension filtered and washed with the same solvent (yield 1·4 g.). When recrystallised from alcohol, the ethylcarbamido-derivative of 8-hydroxyquinoline forms rhombohedral plates which melt at 150° with effervescence.

The *hydrochloride* crystallises from acetone in the form of prisms which melt at 84°.

Analysis. 0·2118 g. gave 0·1184 g. AgCl.
Cl found, 13·83 %; calc., 14·06 %.

Miotic action of synthetic urethanes.

It will be observed that the urethanes described in the preceding section of the experimental portion fall into the following three groups with respect to the position of the basic nitrogen atom: (1) those derived from the isomeric dimethylaminophenols and hence containing the dimethylamino-group attached directly to the benzene ring; (2) those prepared from hordenine with the basic group in a side chain; and (3) those containing a quinoline grouping in which the basic nitrogen atom forms part of a heterocyclic ring. Corresponding with these differences in the nature of the basic groups differences in the basic properties of the urethanes would be expected, and it has, in fact, been found that the hydrochlorides of the first group are, in general, hydrolysed by water to a sufficient extent to yield solutions with an acid reaction but, with the exception of the *N*-phenylurethanes, insufficient to cause the precipitation of the base. It was the discovery that the salts of these urethanes undergo appreciable hydrolysis that led, in the first instance, to the preparation of the urethanes of group two, which, as was anticipated, proved to be stronger bases and hence yielded hydrochlorides which dissolved in water without

appreciable hydrolysis. The urethanes of group three, on the other hand, possess such feeble basic properties that their hydrochlorides are hydrolysed by water to such an extent that the free base is partially precipitated. Since solutions of these quinoline urethanes could therefore only be obtained in the presence of excess of acid they were not considered suitable for pharmacological tests. Experimental work on the physiological activity has, therefore, been confined to the urethanes of the dimethylaminophenol and hordenine series. In both series the hydrochlorides have been employed for this purpose, whilst in the dimethylaminophenol series the methiodides have also been examined. The latter salts were tested partly because, being derived from the strong quaternary ammonium bases, they gave neutral solutions in water, and partly because it was of interest to determine whether the physiological properties of the urethanes underwent any change when the tertiary basic group was converted into a quaternary ammonium group.

A detailed study of the pharmacological properties of these compounds has not, at the present stage of the work, been attempted. On the contrary, they have been examined only with respect to their ability to cause a contraction of the iris when applied to the eye. For this purpose, 5 % solutions of the salts in Ringer's fluid were usually employed. In the cases, however, of the hydrochlorides of the *o*-dimethylaminophenyl esters of methyl- and ethylcarbamic acids and of the *m*-dimethylaminophenyl ester of ethylcarbamic acid, which do not yield solid hydrochlorides, the bases were dissolved in the theoretical amount of 0·115 *N* hydrochloric acid, giving approximately 2 % solutions; while with the methiodide of the *m*-dimethylaminophenyl ester of phenylcarbamic acid and the hydrochloride of the phenylcarbamido-derivative of hordenine, which are only sparingly soluble in Ringer, saturated solutions in this solvent were used. In each case, one drop of the solution so prepared was introduced from a pipette into one eye of a cat. With those compounds which exhibited miotic activity, the pupil commenced to contract in from 10–30 minutes. This effect slowly increased, reached a maximum in about an hour, and persisted to a greater or smaller degree over a period of about 4 hours. The results obtained with the various substances tested are tabulated below:

Substance	Miotic activity
p-$NH(CH_3).CO.O.C_6H_4.N(CH_3)_2$, HCl	+
p-$NH(CH_3).CO.O.C_6H_4.N(CH_3)_2I$	−
p-$NH(C_2H_5).CO.O.C_6H_4.N(CH_3)_2$, HCl	−
p-$NH(C_2H_5).CO.O.C_6H_4.N(CH_3)_3I$	−
m-$NH_2.CO.O.C_6H_4.N(CH_3)_2$, HCl	+
m-$NH(CH_3).CO.O.C_6H_4.N(CH_3)_2$, HCl	+
m-$NH(CH_3).CO.O.C_6H_4.N(CH_3)_3I$	+
m-$NH(C_2H_5).CO.O.C_6H_4.N(CH_3)_2$, HCl	−
m-$NH(C_2H_5).CO.O.C_6H_4.N(CH_3)_3I$	+
m-$NH(C_6H_5).CO.O.C_6H_4.N(CH_3)_3I$	−
o-$NH(CH_3).CO.O.C_6H_4.N(CH_3)_2$, HCl	+
o-$NH(CH_3).CO.O.C_6H_4.N(CH_3)_3I$	−
o-$NH(C_2H_5).CO.O.C_6H_4.N(CH_3)_2$, HCl	+
o-$NH(C_2H_5).CO.O.C_6H_4.N(CH_3)_3I$	−
p-$NH(CH_3).CO.O.C_6H_4.CH_2.CH_2.N(CH_3)_2$, HCl	+
p-$NH(C_6H_5).CO.O.C_6H_4.CH_2.CH_2.N(CH_3)_2$, HCl	−

The application of the substances with miotic activity to the eye of a cat did not usually produce any effect beyond their action on the iris. Occasionally, however, they also produced salivation. This can no doubt be attributed to the action of the drugs on the salivary glands after passage through the lachrymal duct. Nevertheless, this effect was not a constant one but apparently depended upon the individual cat employed, for a substance which produced salivation in one experiment frequently failed to do so in another although its action on the iris was unchanged.

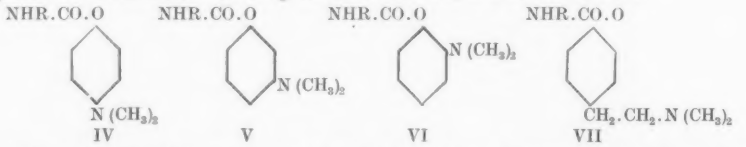
On the other hand, those urethanes which were devoid of miotic activity were found to have an irritant action on the eye, the irritation being accompanied by intense salivation and lacrimation. This was particularly the case with the derivatives of *o*-dimethylaminophenol. In these cases the salivation can probably be attributed to the irritant action of the drugs rather than to a physostigmine-like action.

The miotic action of physostigmine can be readily demonstrated with a 1 % solution of a salt of this base. It will be observed that the solutions of the synthetic urethanes employed in these experiments were considerably more concentrated. An experiment was therefore carried out with a 1 % solution of the hydrochloride of the *m*-dimethylaminophenyl ester of methylcarbamic acid. Miosis was produced in almost undiminished intensity.

Discussion.

A consideration of the possibility, opened by the results recorded above, of the employment of synthetic urethanes in therapeutics must be reserved, and the discussion confined to an analysis of the results with regard to their bearing on the problem of the relationship between chemical constitution and physiological action. In this connection it must be borne in mind that the synthetic compounds have been examined pharmacologically only with respect to one particular property, namely, their action on the iris. While it is probable that the general action on the organism of those urethanes which show miotic activity qualitatively resembles that of physostigmine, at any rate, as regards its selective action on the parasympathetic nervous system, experimental proof that such is the case is at present lacking. Any reference which may be made below to the identity between the physiological properties of the synthetic and natural urethanes must therefore be understood in this limited sense.

In order to facilitate discussion the structural formulae (IV–VII) of the synthetic urethanes, excluding those not tested, are shown below:



IV V VI VII

In each formula R represents a methyl or phenyl group. In IV, V and VI it may also represent an ethyl group and in V a hydrogen atom.

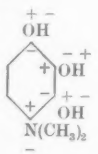
When R is a methyl group, all the compounds resemble physostigmine in physiological properties, and a comparison of the formulae shows that their structures possess two features in common with the alkaloid: they are all substituted phenyl esters of methylcarbamic acid, and they all contain a basic group. It is evident that it is the aromatic urethane grouping which confers miotic properties on these compounds. Whether the basic group is essential for activity is not at present known. Nevertheless, it is certain that the intensity of the activity varies with the position of this group, and it is therefore probable that it not only serves the function of permitting soluble salts to be prepared but also modifies the properties of the urethane grouping in a manner essential to its activity.

That the urethane grouping is itself susceptible to certain changes without completely losing its power of conferring miotic properties on these compounds is also clear from these experiments. Thus, the *m*-dimethylaminophenyl ester of carbamic acid (R = H in V) and the *o*-dimethylaminophenyl ester of ethylcarbamic acid (R = C_2H_5 in VI) both produce a contraction of the pupil. Nevertheless, the general effect of such substitutions is to diminish the activity of the compounds. While, from the nature of the tests employed, it would not be legitimate to make this statement on the grounds that the activities of the two compounds mentioned were actually observed to be less than those of the corresponding methyl urethanes, its validity is evident from the fact that the *p*- and *m*-dimethylaminophenyl esters of ethylcarbamic acid (R = C_2H_5 in IV and V) were completely inactive under the same experimental conditions. The effect of the phenyl group is difficult to determine owing either to the extent to which salts of the *N*-phenylurethanes are hydrolysed or to their slight solubility in water. No activity has been observed with those compounds tested, but the possibility that some *N*-phenylurethanes may possess activity cannot be definitely excluded. Of the synthetic urethanes examined, those which, like physostigmine, are derivatives of methylcarbamic acid thus appear to be the most active miotics.

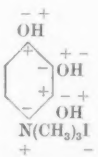
The influence of the basic group must now be considered in greater detail. That this may be attached directly to the benzene ring, or present in a side chain or a heterocyclic ring, as in physostigmine, is obvious from the experiments described above. Beyond this general result, however, a comparison of different types of compounds of different molecular composition can give little information concerning the influence of the basic group. It is difficult to decide, in such cases, what portion of any observed differences in activity is attributable to differences in the position of the basic group and what is to be ascribed to other differences in structure. This difficulty disappears when isomeric compounds of the same type are compared; it is, indeed, largely for this reason that the work here recorded deals mainly with such isomerides. The results obtained with the isomeric dimethylaminophenyl esters of methylcarbamic acid do not, however, yield very much information on this point. No striking difference was observed between the activities of these isomerides,

although the impression was gained that the *p*-compound is somewhat less active than either the *o*- or *m*-compound. The results with the corresponding esters of ethylcarbamic acid are much more definite. Of these, both the *p*- and *m*-compounds, in contrast to the *o*-compound, are completely inactive. Combining the results obtained with the two series of isomerides the author feels justified in concluding that the dimethylamino-group modifies the activity of the urethane grouping in such a way that the activities of the isomerides are in the order $o > m, p$. In any case, it is evident that the basic group exerts a distinct influence on the urethane grouping and that this influence varies according to its position relative to the latter.

When the tertiary basic group is converted into a quaternary ammonium group, a remarkable change, which is in the same direction in both series of isomerides considered in the preceding paragraph, is produced in the activities of these compounds. In the *N*-methylurethane series, the activity of the *m*-isomeride is unmistakably increased, whilst that of the *o*- and *p*-compounds is apparently abolished. Similarly, in the *N*-ethylurethane series the *p*-compound remains inactive, the *o*- loses its activity, whilst the *m*-isomeride, although previously inactive, now manifests distinct activity. The effect of transforming the tertiary basic group into a quaternary ammonium group is thus to diminish the activity of the urethane grouping when the basic group is in the *o*- or *p*-position to the latter, and to intensify this activity when it is in the *m*-position.



VIII



IX

The contrast between the physiological properties of the *o*- and *p*- isomerides, on the one hand, and the *m*- on the other, immediately recalls the differences which are observed between the reactivities of the position isomerides of certain di-derivatives of benzene. In attempting to interpret this resemblance it is convenient to employ the notation of the polarity theory. According to the latter the electronegative dimethylamino-group in dimethylaminophenol would induce the polarities indicated in formula VIII, which may be regarded as representing either of the isomerides. The effect of the positive quaternary ammonium group is similarly indicated in formula IX. Of the three dimethylaminophenols the phenolic group of the *o*- and *p*-compounds should thus be less strongly acidic than that of the *m*-isomeride, whilst the reverse should be the case with the corresponding quaternary salts. Now, except in so far as it has been impossible to differentiate with certainty between the activities of the urethanes derived from *m*- and *p*-dimethylaminophenol, it is, in each series of isomerides, precisely with those urethanes

derived from phenols which, on the polarity theory, are least acidic that the miotic properties are the most pronounced. It should be pointed out that the polarities indicated in formula VIII can only be assigned to the free bases and that, in dealing with salts, it would be necessary to reverse them. Since, however, all the salts of the corresponding urethanes undergo appreciable hydrolysis in aqueous solution, it may be assumed that they react in the organism in the form of the free base.

The fact that the chemical properties, as predicted by the polarity theory, of the phenols from which the urethanes are derived can be correlated with the physiological properties of the latter, indicates that chemical reactivity is one of the factors responsible for their activity. The precise nature of this chemical reactivity has not yet been determined with certainty. Nevertheless, it should be pointed out that the miotics described above are readily susceptible to hydrolysis. This can be shown by boiling an aqueous solution of one of the methiodides. Hydrolysis takes place with such rapidity that the odour of the isocyanate is almost instantaneously detectable. Since the ease with which hydrolysis would take place would undoubtedly vary with the degree to which the phenolic moiety of the molecule possessed acidic properties, it appears reasonable to assume that the physiological properties are dependent on, and vary in some way with, the rate at which the urethanes are hydrolysed. It is conceivable that one of the products of hydrolysis, liberated after adsorption of the urethane by the nerve or nerve ending, might be responsible for the activity of these compounds. Relatively difficultly hydrolysable urethanes would then be inactive on account of the slowness of the formation of the active substances, whilst those which were hydrolysed with extreme ease would be decomposed into surface inactive constituents before reaching the site of their activity.

It is possible that the suggestions made above are applicable to other substances (*e.g.* the tropeines and cocaines) containing easily hydrolysable groups. Such a mechanism would explain the differences in the activities of the enantiomorphic hyoscyamines, if it be assumed that hydrolysis takes place under the influence of enzymes.

Summary.

1. The miotic action of physostigmine (eserine) has been shown to be mainly due to the urethane grouping which is present in the molecule.

2. A number of urethanes, the properties and methods of preparation of which are described, have been synthesised and, where possible, tested for miotic activity.

3. The active urethanes include the isomeric dimethylaminophenyl esters of methylcarbamic acid, the *m*-dimethylaminophenyl ester of carbamic acid, the *o*-dimethylaminophenyl ester of ethylcarbamic acid, and the methylcarbamido-derivative of hordenine. The *m*- and *p*-dimethylaminophenyl esters

of ethylcarbamic acid are inactive, as also are those esters of phenylcarbamic acid which were tested.

4. The basic group modifies the action of the urethane grouping and is probably essential to its activity.

5. Conversion of the tertiary basic group present in the urethanes derived from the isomeric dimethylaminophenols into a quaternary ammonium group exerts a profound influence on the activity of these compounds. The activities of the *m*-compounds are intensified, whilst those of the *o*- and *p*-compounds are abolished.

6. In each series of isomeric urethanes, those compounds derived from phenols which, according to the polarity theory, should be the most acidic are the least active.

7. This correspondence between the alternation in physiological and chemical properties suggests that chemical factors, as distinguished from physical properties, are at least partly responsible for the activity of the compounds.

The author desires to acknowledge his indebtedness to the late Professor A. R. Cushny, F.R.S., for facilities in his department for carrying out the pharmacological tests, and to thank Professor G. Barger, F.R.S., for his interest in the work.

The expenses of this investigation have been defrayed by grants from the Earl of Moray Research Fund of this University.

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XCV. THE TYROSINASE-TYROSINE REACTION.

V. PRODUCTION OF *l*-3·4-DIHYDROXY-PHENYLALANINE FROM TYROSINE.

By HENRY STANLEY RAPER.

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(*Received June 25th, 1926.*)

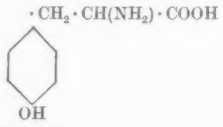
It has been shown by Raper and Wormall [1923] and Raper and Speakman [1926] that when tyrosine is oxidised by tyrosinase, the first visible change is the production of a red pigment. This red pigment is very unstable and changes spontaneously to a colourless substance which in turn undergoes oxidation in the air to produce melanin.

The exact nature of the chemical reactions accompanying these various changes has not yet been elucidated but it has been shown by Raper and Wormall [1925] that deamination of the tyrosine does not take place. It has also been shown by Happold and Raper [1925] that when tyrosinase acts on certain phenols it is probable that ortho-quinones are formed as intermediate products of the reaction and, since tyrosine contains a phenolic hydroxyl group, it would be expected that it also might yield an ortho-quinone under the influence of tyrosinase.

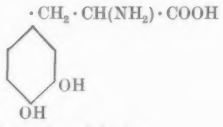
In the present paper it is shown that *l*-3·4-dihydroxyphenylalanine is produced when tyrosine is oxidised by tyrosinase, this substance having been isolated in small amount as one of the products of the reaction. It has been obtained from the solution which results when the red pigment has been converted into the colourless substance, either on standing *in vacuo*, or by boiling, or by standing at the ordinary temperature in the presence of sulphurous acid. This does not enable it to be decided definitely whether the dihydroxyphenylalanine thus isolated is a product of the decomposition of the red substance or is produced continuously from tyrosine by the action of the enzyme, but it provides satisfactory evidence that one point of attack in the tyrosine molecule, when it is oxidised by tyrosinase, is the ortho-position to the phenolic hydroxyl group. It also provides further evidence, if it were needed, that deamination of the side chain does not take place.

It is easily demonstrated that tyrosinase oxidises 3·4-dihydroxyphenylalanine more rapidly than it oxidises tyrosine. Both substances when oxidised by the enzyme give rise to unstable red pigments which, to judge by their properties, are identical. This fact coupled with the isolation of 3·4-dihydroxyphenylalanine as a product of the action of the enzyme on tyrosine suggests very strongly that the initial product of the oxidation of tyrosine is 3·4-di-

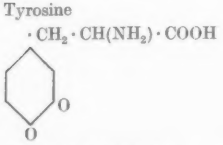
hydroxyphenylalanine and that this is converted by further oxidation into the red substance. The observations of Happold and Raper [1925] indicate that the dihydroxyphenylalanine would yield an ortho-quinone and it is probable therefore that the red substance is produced from this. The red substance is not a quinone as it does not give the guaiacum reaction, liberate iodine from potassium iodide or react with aniline. It is suggested therefore that the following are the stages of the reaction up to the production of the red pigment:



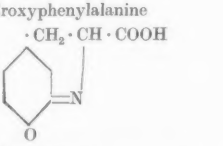
Tyrosine



3·4-Dihydroxyphenylalanine



3·4-Quinone of phenylalanine



Red substance[1]

The quinonoid configuration for the red substance suggested in this scheme would be in conformity with its colour and instability, and its change into the colourless precursor of melanin is probably brought about by an intramolecular change in which a more stable cyclic compound is formed which no longer possesses the quinonoid structure. In favour of this view is the fact that melanin contains almost the same percentage of nitrogen as tyrosine [Raper and Wormall, 1925] and, as is shown in the experimental part, when the colourless substance has been produced from the red pigment, a greater part of the nitrogen which the solution contains is present in the non-amino-form. The question cannot be finally settled until the constitution of the colourless substance has been determined[2].

It has been suggested by Onslow [1923] that one component of tyrosinase is an enzyme (oxygenase), which catalyses the oxidation of ortho-dihydroxy-compounds to peroxides, the latter then serving to yield active oxygen under the influence of a peroxidase, which is another component of the enzyme. 3·4-Dihydroxyphenylalanine would serve as a substrate for this purpose and Onslow and Robinson [1925] have recently advanced the view that it is the initial product of the oxidation of tyrosine and that its continuous production is essential to the further progress of the reaction. If this be true, then its addition to a tyrosinase-tyrosine system should lead to an initial increase in the velocity of oxidation of the tyrosine. The effect would only be noticed in the early stages, for, on the assumption as to the various stages of the reaction put forward above, once the reaction has started, dihydroxyphenylalanine

[1] Alternatively the N may attach itself in the *para*-position.

[2] [Note added July 30th.] That the colourless substance is an indole derivative has been established, but its constitution is at present uncertain.

would be continuously present in the solution. The experiment has been tried and an initial increase in the velocity of the oxidation of tyrosine has been observed.

Incidentally, it may be pointed out that the observations in the present paper disprove the contention of McCance [1925] that *o*-dihydroxy-compounds are not formed in the tyrosinase reaction. McCance states that since catechol compounds combine with boric acid, to yield strongly acid substances, a borate buffer ought to inhibit the oxidation of tyrosine if 3·4-dihydroxyphenylalanine were an essential intermediate product of the reaction, whereas it does not. This view does not take cognisance of the fact that only traces of 3·4-dihydroxyphenylalanine would be present at any one time in the tyrosinase reaction and therefore any change in the p_H of the solution would be small, also that it is probable that the boric acid compound is slightly dissociated in solution and therefore free dihydroxyphenylalanine would be present. Actual experiment shows that 3·4-dihydroxyphenylalanine is readily oxidised by tyrosinase when it is added to a borate buffer with a reaction of p_H 8.

The production of 3·4-dihydroxyphenylalanine from tyrosine by a biological method of oxidation overcomes an obstacle which previously existed in accepting the view that tyrosine is the parent substance of adrenaline. The presence of an enzyme in the suprarenal gland which oxidises tyrosine has not yet been demonstrated but it is probable that 3·4-dihydroxyphenylalanine may be produced elsewhere in the body. Bloch [1917] and Bloch and Schaaf [1925] have presented evidence that it is the only substrate which is effective for the production of melanin in epithelial pigment cells by "dopa" oxydase. This enzyme does not attack tyrosine but its mere existence and its apparent specificity render the production of 3·4-dihydroxyphenylalanine in some situation in the body very probable.

Experimental.

Preparation of the enzyme.

The enzyme used in the experiments described in this paper was prepared from mealworms (*Tenebrio molitor*) by a modification of Gortner's process [1910]. About 100 g. mealworms are killed with chloroform water, washed and pounded in a mortar with successive portions of chloroform water until only the hulls remain. The resulting fluid is strained through coarse muslin to remove remnants of the hulls and at this stage measures about 400 cc. This is filtered and the residue on the filter paper washed with chloroform water containing a trace of acetic acid until the filtrate is colourless and gives only a slight turbidity with lead acetate and ammonia. This process usually occupies about 10 days because filtration is slow. The residue on the filter paper is now scraped off, ground up in a mortar with small amounts of chloroform water[1] and the whole transferred to a wide-mouthed stoppered bottle. A few cc. of

[1] In all about 400 cc.

chloroform are added and then ammonia until the reaction is slightly alkaline to litmus. With occasional shaking the enzyme gradually becomes very finely dispersed and is an extremely active preparation of tyrosinase. It retains its activity in this condition for many weeks. If the solution be acidified slightly with acetic acid, the enzyme is completely precipitated in an easily filterable condition.

Isolation of l-3·4-*dihydroxyphenylalanine.*

1 g. of tyrosine is dissolved by boiling in 500 cc. of water and the solution diluted to 2 litres. By cooling, the temperature is adjusted to about 27° and 100 cc. of the enzyme preparation described above are added. By addition of very dilute acetic acid the reaction is adjusted to between p_H 6 and 6·5. The solution is placed in a 2½ litre bottle and oxygen bubbled through until the air above the liquid has been largely displaced by oxygen. The bottle is now stoppered and well shaken. The shaking is repeated frequently and about every hour the oxygen is renewed by a repetition of the initial bubbling. The liquid quickly becomes red and usually after 2–4 hours, depending on the temperature and the activity of the enzyme, the enzyme has separated out as a flocculent precipitate. This generally happens when about 50–60 % of the tyrosine has been oxidised and is apparently due to a reaction between the enzyme and some product of its action on tyrosine. To ensure complete precipitation, 10 cc. of 1 % acetic acid are added, and the solution filtered rapidly, using several large funnels. The filtrate is clear and has a fine red colour resembling diluted, laked blood. The red substance may now be rapidly converted into the colourless melanin-producing substance by boiling in an inert atmosphere and rapidly cooling out of contact with air, or more slowly by keeping the solution *in vacuo* for a few days or by adding 30 cc. of saturated sulphurous acid and allowing it to stand for 48 hours in a stoppered bottle. The last method has been the one most frequently used because of its simplicity. When the transformation of the red substance is complete the liquid has a pale brownish colour. If sulphurous acid has been added it must be removed at this stage by boiling and then cooling rapidly. The next step is the removal of the colourless precursor of melanin. 40 cc. of 20 % lead acetate are added and the solution freely exposed to air or shaken periodically in a large bottle. A whitish precipitate, consisting partly of lead sulphate, but also containing some of the melanin-producing substance, is formed when the lead acetate is added and this gradually darkens in the air. The solution is allowed to stand with occasional shaking for 48 hours to allow for complete precipitation of melanin and is then filtered. To the colourless filtrate 1 % ammonia is added until the liquid is faintly but distinctly alkaline to litmus. An excess of ammonia must be avoided as this throws down tyrosine, which is still present, as well as 3·4-dihydroxyphenylalanine. The liquid is allowed to stand and the precipitated lead compound of dihydroxyphenylalanine washed once by decantation. It is then redissolved by adding dilute acetic acid and the

precipitation with lead acetate (10 cc.) and ammonia repeated. After again washing by decantation, the precipitate is filtered off by suction on a wad of paper pulp and washed several times with water. The pulp and adherent precipitate are transferred to a wide-mouthed, stoppered bottle, well shaken with 150 cc. water and decomposed with hydrogen sulphide. The pulp and lead sulphide are filtered off, washed, and the filtrate and washings concentrated to small bulk by distillation *in vacuo*. By cautious addition of ammonia, the solution is partially neutralised until it is acid to litmus but not to Congo-red. It is then clarified by boiling with charcoal, a trace of sulphurous acid is added and it is taken to dryness in a vacuum desiccator. The residue is extracted with a small amount of hot water and filtered to remove a small amount of insoluble inorganic matter. On again concentrating in a vacuum desiccator, dihydroxyphenylalanine crystallises out. At this stage it is best to work up the products from at least 2 g. of tyrosine because of the small yield. With 2 to 3 recrystallisations from water containing a trace of sulphurous acid the product is obtained pure. It crystallises, as observed by Guggenheim [1913], in thick prisms or fine needles according to conditions. From 2 g. of tyrosine 0·034 g. of the pure substance was obtained, M.P. 279° (corr.) with decomposition. Mixed with 3·4-dihydroxyphenylalanine from *Vicia faba*, the M.P. remained unchanged.

I am indebted to Dr F. R. Goss for a micro-analysis.

Found, C, 54·5 and 54·8 %; H, 5·8 and 5·7 %; N (Dumas), 7·1 %.

Calculated, C, 54·8 %; H, 5·6 %; N, 7·1 %.

The product gave a green colour with very dilute ferric chloride which was changed to purple by ammonia. It reduced ammoniacal silver nitrate at once in the cold. Treated with dilute sodium carbonate it gave a yellow solution which gradually became brown and then brownish black in the air. These properties and the M.P. are identical with those of the naturally occurring compound in *Vicia faba*. Since the naturally occurring tyrosine was used in the preparation, it was expected that the laevorotatory form of the compound would be obtained by the action of tyrosinase. The M.P. already indicated that this was so, inactive 3·4-dihydroxyphenylalanine having a M.P. of 263–272° [Funk, 1911]. To confirm this, 20 litres of a saturated tyrosine solution were oxidised by tyrosinase and the dihydroxyphenylalanine obtained by the method described above was examined polarimetrically. 0·155 g. in 9·94 cc. *N* HCl gave a rotation in a 1 dm. tube of $-0{\cdot}17°$ which gives $[\alpha]_D^{19°} = -11{\cdot}0°$. Using more concentrated solutions, Guggenheim [1913] and Waser and Nevandowsky [1921] obtained values of $-14{\cdot}28°$ and $-12{\cdot}74°$ respectively, for the naturally occurring substance. The optical activity of the dihydroxyphenylalanine may be adduced as additional evidence, if it were required, that it is not formed from tyrosine by a deamination process with subsequent addition of ammonia.

Oxidation of 3·4-dihydroxyphenylalanine by tyrosinase.

The 3·4-dihydroxyphenylalanine used was prepared from *Vicia faba* by Guggenheim's method. 50 mg. were dissolved in 95 cc. of a phosphate buffer, p_H 6, 5 cc. tyrosinase were added and oxygen bubbled through the solution which was placed in a stoppered bottle. The solution was shaken frequently. It rapidly became red, and after an hour was very deeply coloured (under the same conditions it was judged that tyrosine would have taken at least ten times as long to produce the same depth of colour). The solution was filtered through a thick wad of paper pulp to remove the enzyme. 5 cc. of the filtrate were placed in a Thunberg tube and 1·03 cc. *N*/5 NaOH added to make the reaction approximately p_H 8. A control tube with 5 cc. filtrate and 1·03 cc. water was set up for comparison. Both tubes were immediately evacuated, placed in a bath at 37° and the colour changes observed. The tube at p_H 8 had lost its red tint after 15 minutes whereas that at p_H 6 was still a deep red. On admitting air to the tube at p_H 8 and shaking it blackened immediately. This behaviour is exactly like that described by Raper and Speakman [1926] for the red substance produced from tyrosine. The filtrate on boiling also decolorised rapidly like the red substance from tyrosine. It was not changed by adding aniline water. A small amount of dihydroxyphenylalanine was also oxidised by tyrosinase at p_H 6 in the absence of a phosphate buffer. When the red colour had developed, the solution was filtered and the filtrate boiled to decolorise it. On cooling and adding a drop of dilute ferric chloride the typical green colour given by dihydroxyphenylalanine was obtained but this then rapidly changed to a bluish black. This again is similar to the behaviour of the red substance prepared from tyrosine except that with the latter only a trace of green is obtained, owing to the very small amounts of dihydroxyphenylalanine present.

These various reactions of the red substance prepared from dihydroxyphenylalanine suggest that it is identical with the red substance obtained from tyrosine.

Nitrogen distribution in the solution after decoloration of the red substance.

A solution of the red substance free from enzyme was obtained from tyrosine as described earlier in this paper. 100 cc. were kept *in vacuo* until the red colour had disappeared and it was then distilled *in vacuo* until about one-third of the solution remained. With portions of this, estimations of total nitrogen, amino-nitrogen and ammonia (Folin) were made. The figures obtained per 100 cc. were: total N, 20·16 mg., amino-N, 9·71 mg., ammonia-N, 2·88 mg. The non-amino-N not accounted for by ammonia was thus 7·57 mg. The ammonia present was due to that contained in the enzyme solution, so that the non-amino-N accounted for 44 % of the total N minus ammonia-N. In a similar experiment in which the red substance was allowed to decolorise in

presence of sulphurous acid, a figure of 41 % non-amino-N was obtained. It was proved (by the bromination method) that in these experiments 57 % of the tyrosine had been oxidised, so that of the amino-N originally present in the tyrosine which had been oxidised, $\frac{44}{57} \times 100$, or 77 % was now present in the non-amino-form. This shows that in the colourless substance which is produced from the red pigment, the nitrogen is probably part of a cyclic structure.

Velocity of oxidation of tyrosine in the presence of dihydroxyphenylalanine.

The method used was that described by Raper and Wormall [1923], the tyrosine being estimated by bromination. A preliminary experiment showed that any dihydroxyphenylalanine present in the samples of the oxidation mixtures withdrawn for analysis was destroyed by the subsequent treatment with alkali which was adopted by Raper and Wormall prior to the bromination. This ensured therefore that tyrosine alone was being estimated in those mixtures to which 3·4-dihydroxyphenylalanine had been added.

The determination of the effect of dihydroxyphenylalanine on the amount of tyrosine oxidised was carried out as follows.

About 0·15 g. tyrosine was dissolved in a phosphate buffer, p_H 6 (solution A), and a solution of dihydroxyphenylalanine was made containing 12·8 mg. in 15 cc. water (solution B).

Flask 1. 135 cc. solution A + 10 cc. water + 5 cc. enzyme.

Flask 2. 135 cc. solution A + 10 cc. solution B + 5 cc. enzyme.

Flask 3. 135 cc. buffer solution + 10 cc. water + 5 cc. enzyme.

Temperature 17·5°. Immediately after making up each mixture, duplicate 20 cc. samples were withdrawn for tyrosine estimation. The air current was then started and samples were withdrawn after 65, 125 and 205 minutes. The samples as withdrawn were run into flasks containing 0·5 cc. 10 % acetic acid and the estimation carried out exactly as described by Raper and Wormall.

The following figures were obtained:

Time (minutes)	Tyrosine in 20 cc. (mg.)		Tyrosine oxidised per 20 cc. (mg.)	
	Flask 1	Flask 2	Flask 1	Flask 2
0	8·06	7·89	0	0
65	6·91	6·40	1·15	1·49
125	5·62	5·35	2·44	2·54
205	4·52	4·42	3·54	3·47

These results show a 30 % increase in oxidation of tyrosine in the first 65 minutes when dihydroxyphenylalanine is present. After 125 minutes the increase has diminished to 4 %, which is almost within the experimental error of the method, and after 205 minutes no increase is observed.

Oxidation of 3·4-dihydroxyphenylalanine in a borate buffer.

This experiment was designed to test the validity of the conclusions of McCance [1925]. A low concentration of dihydroxyphenylalanine was used purposely, since if the hypothesis which has been advanced in the introduction to the present paper is correct, only a very small concentration of this substance could be present at any one moment owing to its ease of oxidation by tyrosinase. 11 mg. dihydroxyphenylalanine were dissolved in 50 cc. of a borate buffer, p_H 8; 2 cc. of enzyme were added and oxygen bubbled through the solution for a short time. The bottle containing the solution was stoppered and shaken frequently. After 8 minutes the development of a red colour was clearly visible and after 23 minutes the solution was a deep reddish crimson. The colour deepened as the oxidation proceeded and became finally a dark reddish brown. This experiment proves therefore that McCance's conclusions were based on false assumptions.

SUMMARY.

1. *l*-3·4-Dihydroxyphenylalanine has been isolated and identified as a product of the action of tyrosinase on *l*-tyrosine. The yield of the pure substance is not more than 3 % of the tyrosine oxidised.

2. It is suggested that 3·4-dihydroxyphenylalanine is the first product produced in the tyrosinase-tyrosine reaction and that this on further oxidation produces the red pigment which is characteristic of the reaction. A probable formula for the red pigment is given.

3. In the presence of a small amount of 3·4-dihydroxyphenylalanine the rate of oxidation of tyrosine by tyrosinase is initially increased. This supports the views of Onslow on the mechanism of action of tyrosinase.

I wish to express my thanks to Mr T. Kirkman for assistance in some of the experiments, and to the Government Grant Committee of the Royal Society for a grant in aid of the expenses of this work.

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XCVI. STUDIES IN CREATININE AND URIC ACID METABOLISM.

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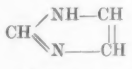
(*Received June 28th, 1926.*)

Introduction.

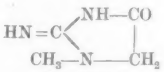
In regard to the origin of urinary creatinine, the theory most generally accepted at present is that creatinine arises from creatine, but it is only the recent work of Benedict and Osterberg [1923] which has provided definite evidence in favour of this view and their work still requires confirmation.

Folin still maintains the essential independence of the two and Mellanby holds the view that creatinine is formed in the liver from substances brought to it by the blood stream. On the whole, the evidence does not enable one to come to any definite and final conclusion in regard to the origin of urinary creatinine.

The idea underlying the following work was that creatinine might arise from some analogous ring compound. Mellanby [1908] writes: "From a chemical consideration it is more probable that the ring formation of creatinine comes direct from a tissue breakdown and that this ring is then hydrated to a creatine chain by muscle rather than that the creatine chain is dehydrated to form the creatinine ring." Creatinine may be regarded as a derivative of glyoxaline or iminazole.



Iminazole



Creatinine

The iminazole ring points to relations with other iminazole derivatives such as histidine and the purines. The results below show the effects on urinary creatinine of ingestion of uric acid in man and in the dog.

Experimental methods.

(*a*) The subjects of the experiments were young men, students and laboratory workers. During the period of experiment the diet of each individual was as free from purine and creatine as possible. No meat was eaten and on the whole no animal food except eggs, milk and occasionally fish. Besides this the diet consisted of potatoes, cabbage, peas, etc. and bread and butter. There was no restriction in regard to drinking tea or coffee as these beverages contain

too small an amount of purine (caffeine) to affect the excretion of uric acid to any appreciable extent.

The urine was collected for exact 24-hour periods. A few drops of chloroform in each collecting bottle acted as a preservative. The periods ended at 9 a.m. and the creatinine and uric acid were estimated as soon as possible afterwards.

Creatinine was determined by means of Folin's colorimetric method. The picric acid used was purified by crystallisation from benzene as described by Benedict [1922]. The colorimeter was placed in a specially constructed dark box and the source of light, a daylight lamp, ensured a constant illumination.

Uric acid was estimated by Cole's modification of the Hopkins method [1926].

(*b*) In the experiments on a dog, the animal was kept in a metabolism cage and fed on dog biscuits, porridge and milk. The urine for 48-hour periods was collected in bottles containing a few cc. of toluene.

At the end of each 2-day period the sides and funnel-shaped bottom of the cage were washed with water, the washings added to the urine and the whole made up to 2 litres. The clear filtrate from 200–300 cc. of the diluted urine was used for the estimation of creatinine and allantoin.

Allantoin was estimated by the Wiechowski method as described by Hunter and Givens [1911].

Ingestion of uric acid in man.

As soon as the daily elimination of uric acid and creatinine had reached a fairly steady level, 2–5 g. uric acid suspended in water were taken by mouth in two or three doses during the course of the day. An increased output of uric acid over the previous steady level indicated that absorption of uric acid had taken place. This increased excretion of uric acid after ingestion did not always take place and this effect varied from individual to individual and also in the same individual at various times.

In every case, however, in which the uric acid had been absorbed a large increase in creatinine excretion resulted.

Subject A. R. (Fig. 1) took 3 g. of uric acid on the 7th day. On the following day the excretion of uric acid rose from an average value for the preceding 7 days of 0·38 g. to 0·634 g., an increase of 0·254 g. The creatinine excretion for the same day rose to 2·49 g., an increase of 0·63 g.

A second dose of 3 g. was taken on the 14th day and in this case the uric acid seemed to have been absorbed more rapidly as there was an increase both in the uric acid and creatinine outputs for that day.

A similar result was obtained with G. L. B. (Fig. 2). This subject showed rather a peculiar fall in creatinine excretion on Sundays. (This low value was always present on this day. 1st and 8th periods, Fig. 2.) A subsequent

experiment on the same subject showed exactly similar low outputs of creatinine on the 1st and 8th periods.

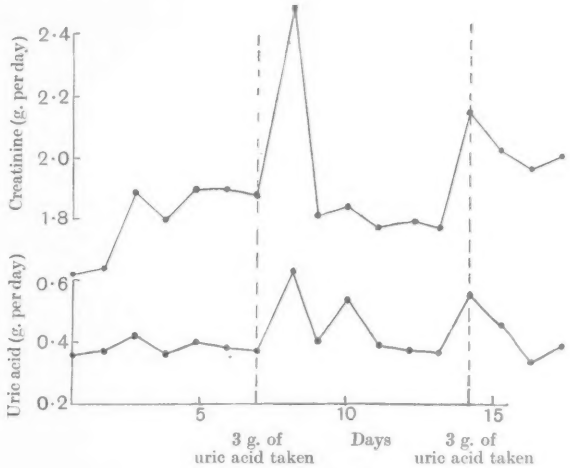


Fig. 1. Subject A. R.

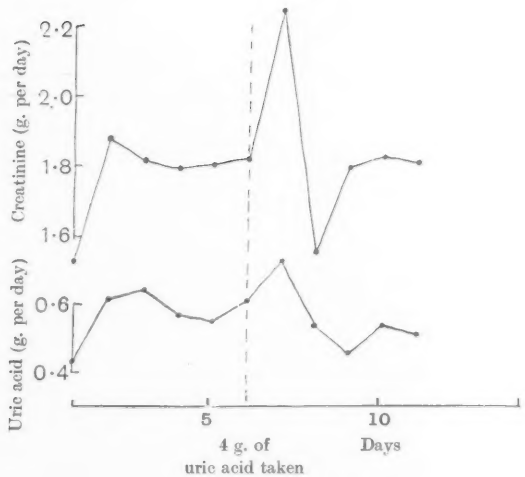


Fig. 2. Subject G. L. B.

Two experiments, one on G. L. B. (Fig. 2) and one on R. W. (Fig. 3), were run concurrently and show rather strikingly the difference whether uric acid is absorbed or not. In the former case after taking 4 g. uric acid the amount

excreted in the urine rose from an average value of 0·55 g. in the fore-period to 0·72 g. and the creatinine increased from 1·80 to 2·25 g. In the second case no change occurred in the uric acid and the creatinine also remained at the previous steady level. On taking a second dose of 4 g. some days afterwards the same result was obtained. Two experiments on subject A. R. also illustrate this point. The first dose of uric acid (3 g. on the 5th day) caused a rise both in the creatinine and uric acid, while the second dose (4 g. on the 14th day) caused no change in either.

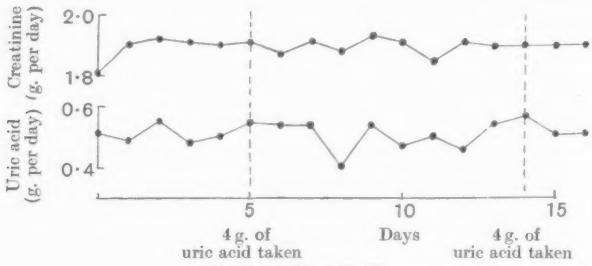


Fig. 3. Subject R. W.

Similar though less well-marked results were obtained in the case of subject H. Z.

Table I summarises some of the results obtained.

Table I.

Subject	Amount of uric acid ingested (g.)	Increase in uric acid (g.)	Increase in creatinine (g.)
A. R.	3	0·23	0·63
A. R.	3	0·16	0·35
A. R.	4	—	—
G. L. B.	4	0·17	0·42
R. W.	4	—	—
H. Z.	4	—	—
H. Z.	2	0·06	0·18

From the above results we may thus conclude that ingestion of uric acid when followed by absorption causes in all cases an increase in the excretion of creatinine.

An explanation of the increased creatinine excretion after uric acid ingestion may be found in a consideration of the different results obtained when purine-rich material is given and when uric acid is ingested as such. In the latter case an increase in creatinine is obtained, while with purine-rich food no such effect is shown. Both after thymus and pancreas ingestion the uric acid output rose while the creatinine remained at its previous steady level. Taylor and Rose [1913] obtained the same result on feeding sweetbreads: the increase in uric acid was not accompanied by an increase in creatinine excretion.

The ingestion of uric acid as such may cause its appearance in the blood in a much more insoluble and less easily excreted form than when it appears as the result of purine-rich food. The kidney normally gets rid of uric acid less readily than creatinine or urea and transformation of this less easily excreted form of uric acid to creatinine and urea may be an additional means by which the body gets rid of the excess of uric acid. When purine-rich food is given, the uric acid may appear in blood in the normal more easily excreted form and hence there is no need for a transformation to creatinine.

The above interpretation although not improbable can by no means be regarded as conclusive until we know more about the form in which uric acid is present in blood.

Ingestion of uric acid in the dog.

On reference to Table II it will be seen that in the dog the amount of creatinine excreted daily is less than the daily allantoin excretion. In the human subject we find just the reverse relation between the amounts of the two endogenous catabolites in the urine.

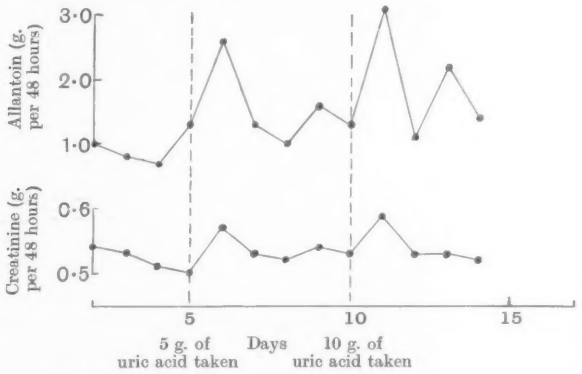


Fig. 4. Subject dog I.

The uric acid, 5 g. on the 5th day and 10 g. on the 10th day, was given mixed with milk and porridge. The average 48-hour period allantoin output for this dog was 1·23 g. The first dose of uric acid caused an increased excretion of allantoin of 1·35 g. and the second an increase of 1·88 g. (see Fig. 4). In both cases there was a secondary rise in the allantoin excretion a day or two after the first rise—0·41 g. and 0·97 g. respectively. So that 5 g. uric acid caused in all an increased elimination of 1·76 g. allantoin and 10 g. gave rise to an increased output of 2·85 g., *i.e.* from 30 % to 35 % of the uric acid ingested appeared as allantoin. Only traces of uric acid were found in the urine. Throughout this experiment the creatinine output remained fairly constant. This is in marked contrast to the effect on creatinine excretion of uric acid

ingestion in man. Now, the dog's organism contains the enzyme uricase and thus possesses an excellent means whereby thoroughly and rapidly to get rid of any uric acid which may be introduced. In the dog therefore there is this natural path for the speedy removal of uric acid, whereas this means of dealing with uric acid is lacking in the human organism which, if the theory put forward above is correct, is forced to a certain extent to turn the less easily excreted uric acid into the more easily excreted creatinine and urea.

Allantoin-creatinine ratio in various animals.

The animals were all kept on a diet free from creatinine and purine. In the case of the rats and guinea-pigs, four animals were kept in a small metabolism cage for 6 days, at the end of which period 200–400 cc. urine had been collected. The dogs and rabbits and cat were kept in a large metabolism cage for from 2–4 days according to the size of the animal and amount of urine excreted. In each case the urine was made up to 1 or 2 litres with water according to amount of urine collected. 200 cc. were filtered and the filtrate used for determinations.

Table II.

Animal	Weight (kg.)	Allantoin (mg.)	Creatinine		Allantoin / Total creatinine
			Total (mg.)	Preformed (mg.)	
Rat I	0·25	20	4·6	2·6	4·35
Guinea-pig	0·31	76	17·5	9	4·34
Dog IV	1·6	273	59	39	4·63
Rabbit	2	221	—	86	2·57
Dog II	6·3	286	—	111	2·58
Dog I	8·5	615	—	261	2·35
Dog III	18·5	1278	—	538	2·37
Cat	1·9	209	—	51	4·01
Coyote	7	870	—	270	3·2

The figures for the coyote are taken from Hunter and Given's paper [1911]. In this case the creatinine and allantoin were determined when the animal was on a meat diet. It is interesting to find that in this animal also the amount of allantoin excreted is more than the amount of creatinine. Total creatinine determinations (creatine + creatinine) were carried out on the urines of rats, guinea-pigs and dog IV. The amount of creatine in the urine of the other animals was small.

From Table II it will be seen that the smaller animals all show a ratio of allantoin to total creatinine of about 4·4 to 1, while the ratios of allantoin to preformed creatinine in all the other animals except the cat are in the neighbourhood of 2·4 to 1. The rats and guinea-pigs show ratios which are nearly the same. The weight of dog III was a little more than double that of dog I, yet the allantoin-creatinine ratios are practically identical.

Results and discussion.

Before proceeding to a discussion of the results of ingestion of uric acid some interesting points in regard to the normal daily outputs of creatinine and uric acid must be dealt with.

It will be noticed (see figures) that the daily creatinine excretion, although more constant on the whole than the daily uric acid excretion, shows marked variations. This was found to be the case in each of the four subjects of the experiments. There were individual differences in regard to the extent of these variations, subject R. W. showing a more constant daily creatinine output than the other three, but taking into account the whole series of experiments it is evident that the creatinine excretion from day to day is not so remarkably constant as had been supposed. It is thus analogous to the daily endogenous uric acid elimination which was also found to be not so constant in the same individual as was first put forward by Burian and Schur [1905]. Cathcart comes to the same conclusion [1912].

The quantity and kind of purine-free food is of importance in determining the endogenous uric acid output and a similar relation may hold in the case of creatine- and creatinine-free food and the elimination of endogenous creatinine.

Folin's figures [1905] illustrate the same point: the daily variations in the excretion of creatinine are well marked.

A further point of interest is that these daily variations in the creatinine follow fairly closely the variations in the excretion of uric acid. This was the case in the majority of urines analysed. Leathes [1907] studied the hourly output of uric acid and found that on a purine-free diet the excretion of uric acid is most active in the early waking hours and much less active at night, in spite of the fact that the kidneys are more active during sleep than during waking hours of the day, and, even on a low nitrogen diet, are almost at maximum activity at night. The hourly creatinine output shows variations similar to the hourly excretion of uric acid which are of interest in connection with results discussed above. Leathes concludes from his experiments that the output of uric acid and creatinine during the night must be the expression of a diminished functional activity in some other organ or organs than the kidney.

The results obtained all point to the fact that creatinine and uric acid are correlated in some way. It is not improbable that nuclein (*i.e.* histidine-containing protein plus nucleic acid) is a common precursor of creatinine and uric acid.

On the other hand, if we assume that creatinine does arise from creatine, the above results may be explained if we accept Burian's theory of the origin of uric acid [Burian and Schur, 1905]. According to Burian only a small part of the endogenous uric acid is derived from nucleoprotein of cellular origin. He looks upon the hypoxanthine of muscle as the most important source

of uric acid. Hypoxanthine is formed in muscle principally during stimulation, and conversion of hypoxanthine to uric acid goes on continually both while muscle is at rest and during stimulation. Furthermore, Burian states that hypoxanthine formation is analogous to creatine formation in muscle: "Nach meiner Auffassung entstammen das Hypoxanthin und das Kreatin ein und demselben Grundprozesse." Thus if creatinine arises from creatine and uric acid from hypoxanthine we have an explanation of the correlation in the excretions of uric acid and creatinine.

From a more general point of view, this correlation is probably simply an expression of the fact that they are both end-products of endogenous metabolisms.

SUMMARY.

1. The daily excretion of creatinine was found to undergo marked variations and is not so constant as was supposed. The daily variations in the creatinine and uric acid excretions on a diet free from creatine-creatinine and purine followed one another closely.

2. The result of uric acid ingestion in man was that an increase in creatinine excretion was obtained whenever absorption of uric acid had occurred. This probably points to a transformation of the iminazole nucleus in uric acid to creatinine.

3. Ingestion of nuclear-rich material (thymus and pancreas) caused an increase in the uric acid output but did not affect the creatinine.

4. 5–10 g. of uric acid given to a dog *per os* caused a large increase in the allantoin excretion, the creatinine however remaining at the previous fairly steady level.

5. In man the ratio of uric acid to creatinine is about 1 to 4. On the other hand, in dogs the ratio of allantoin to creatinine is about 2·4 to 1. Ratios similar to the latter were found in other animals (cat, rat, guinea-pig, rabbit).

I wish to express my sincere appreciation of Mr A. D. Ritchie's invaluable assistance and advice during the course of this work, and also to thank Messrs G. L. Brown and R. Whitehead for offering themselves as subjects for various experiments from time to time.

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XCVII. THE DIETETIC VALUE OF OATMEAL PROTEINS.

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Proteins of vegetable origin, on the whole, are regarded as being of lower biological value than those from animal sources, and even if the vegetable protein be a good one, there is usually such a small amount present that it is of little use in contributing to the protein ration of man. Thus the biological value of potato protein is 79 [Karl Thomas, quoted by Lusk, 1917] but the amount present is only 1·6 % in new, and 2·1 % in old potatoes [Plimmer, 1921].

The percentage of protein in oatmeal varies according to different authorities; Plimmer [1921] gives 11·9 %, Bailey [1921] 14·7 % and Sherman [1924] 16·1 % of protein. Taking even the lowest figure, oatmeal appears to be a rich source of protein compared with vegetables and fruits. Research on oatmeal protein has a varied history. The earlier observations of American workers showed oat protein to be of poor quality, but their later work caused them to modify this opinion. Osborne and Mendel [1918] fed rats on protein concentrates from various cereals and found that the young animals failed to grow adequately on the oat diet. In more recent experiments these observers [1920] used the entire cereal grains (finely ground) and found that, with the addition of salt mixture and butter fat, a satisfactory diet was produced, and some of the animals grew to a large size indicating that the oat protein was adequate in meeting the needs of the rat. They state, however, that the animals did not eat the diet well, and suggest that this may be the reason why some of them failed to grow.

McCollum and his co-workers, in their earlier experiments, also found oat protein to be of poor value, but further work evidently altered this opinion, for McCollum [1922] says: "the oat kernel is comparable with wheat or maize in its dietary properties in nearly all respects." The omission of the necessary mineral salts from the diet is recognised as sufficient to account for the failure of the earlier experiments.

Sherman, Winter and Phillips [1919] made interesting, though rather short experiments on human beings. Two subjects were fed on "scones" made from oatmeal, starch and butter, eaten with apple and sugar. In the main experiment, which lasted 12 days, a small quantity of milk was added to the diet.

The two subjects showed N balances of ± 0·0 and + 0·2 g. per day, although they only received sufficient calories to meet their energy requirement and 0·57 or 0·55 g. of protein per kg. of body weight. In another experiment, also of 12 days' duration, but in which no milk was added to the diet, the nitrogen balance was − 0·7 and − 0·6 g. of N per day respectively. These workers therefore conclude that as nitrogenous equilibrium was maintained with such a low protein intake in one series, and so closely approximated to in the other, oatmeal proteins are "efficiently utilised in the maintenance metabolism of healthy adults."

Opinion differs as to the proteins in oats. Sherman [1924] writes: "the proteins of oats have proven particularly difficult to purify, and have therefore not yet been studied so thoroughly as have some of the other grain proteins."

According to Wiley [1917] oats contain three proteins, one soluble in alcohol, one soluble in dilute salt solution, and one soluble in alkali. Bailey [1921] also gives three proteins, avenin or oat myosin, oat legumin or oat casein, and oat gliadin. Plimmer [1920] states that oat protein consists of gliadin and is a little vague as to the presence of a globulin, an albumin or both. Onslow [1920] gives avenalin as a kind of globulin found in oat seeds. By simple qualitative analysis the author has obtained three proteins from oatmeal, two of which are soluble in cold water and the third is insoluble in water, but soluble in strong alkali. Of the two found in the cold water extract, one coagulated on heating and the other did not. At first, therefore, one is tempted to say that a protein of the albumin variety is present, but as suggested by Onslow [1920] it is quite possible that this protein is a globulin and that the seeds themselves contain sufficient salt to provide for the dissolving of the protein. All three proteins gave a positive sulphur test and a positive Millon's reaction, and the coagulable and insoluble ones in addition contained tryptophan. From this simple analysis one would expect that oatmeal should prove a good source of protein in the diet.

Experimental.

Growth.

(*a*) *Diet.* In order to test the efficiency of oat protein, the diet must obviously be complete as regards other food factors. Oatmeal contains carbohydrate, protein, fat, some mineral salts and vitamin B; butter and salt mixture [Hartwell, 1922] were added. It has been shown that cereals are low or deficient in calcium and other mineral constituents necessary for animal welfare, and it was thought better to add salt mixture entire than to try and pick out those elements required to supplement the mineral content of oatmeal, and so to avoid a possible risk of deficiency. Butter was used to supply vitamins A and D. Many dietetic workers may criticise this adversely and say that as oatmeal itself contains fat, it would have been better to use cod-liver oil which is notably rich in vitamins A and D and these factors could

thus have been provided and yet less fat have been added to the diet. The reason for using butter is that this substance is a food eaten by human beings, while cod-liver oil cannot be regarded as an ordinary article of diet.

The proportions used were 100 g. oatmeal, 2·88 g. salt mixture, 14 g. butter and 500 cc. water. Details of preparation are given in an earlier paper [Hartwell, 1925].

(*b*) *Animals and method.* Piebald rats were used in these experiments; they were bred in the laboratory and were given the oatmeal diet from the age of 23 days (2 days after weaning). Five females and 5 males were used; they were kept in one large cage so that they could breed as soon as they were old enough; food was given *ad lib.*, with water to drink. The animals ate the diet readily (Osborne and Mendel [1920] found that some of their rats did not do so, possibly because they gave the oats in an uncooked form). Weighings were made daily at first, but when the animals were older and therefore growing less rapidly, once per week.

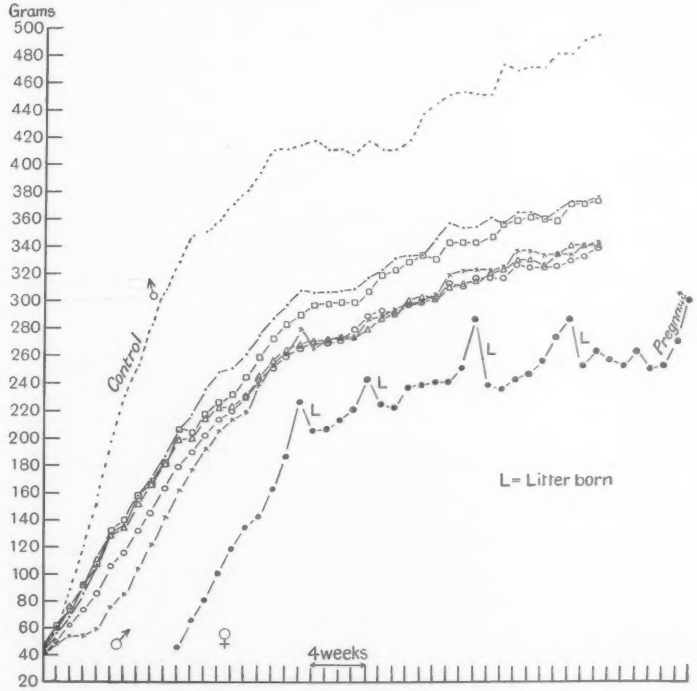

Fig. 1. Growth curves of rats fed on a diet of oatmeal, butter and salt mixture.

(*c*) *Results.* The rats grew well, though not so rapidly as control animals which received a mixed diet of kitchen scraps and bread and milk. The average weight of the five bucks after $8\frac{1}{2}$ months on the oatmeal diet was

353·4 g. as compared with 272·5 g. given by Donaldson [1924] as the weight at a corresponding age. The growth curves of the bucks and one of the does (as examples) are given in Fig. 1.

The animals were fit and well throughout the experiment and their coats were especially thick and glossy. Litters were born, but this is dealt with under lactation.

Lactation.

It is highly probable that the requirements of the animal vary with different phases of its existence; for instance, a diet may be eminently suitable for growth, but quite unsatisfactory for gestation or lactation. The does belonging to the experiment previously described produced litters, but the young were not always reared. This may be due to the unsuitability of the diet for gestation or lactation or both. A second series of experiments was therefore made in which the does received a good mixed diet during gestation and the oatmeal diet was only given after the birth of the litter.

Exp. 1. The oatmeal diet was given from 3 weeks of age.

In some cases the diet was changed during lactation to bread and milk. The results are shown in the following table. When necessary the litters were reduced to 6 on the day after birth.

	Exp. number	Number in litter	Average weight 2nd day (g.)	Number reared	Average weight at weaning (g.)	Diet of mother
Rat 1	1615	8	4·9	0	—	Oatmeal diet
	1651	8	4·9	6	21·5	,,
Rat 2	1621	1+2 dead	4	0	—	,,
	1671	6+1 ,,	4·66	1	20	,,
	1748	4+1 ,,	5	2	21	,,
	1793	7	5·7	1	15	,,
	Litter born dead					
Rat 3	1647	6+1 dead	5·5	0	—	,,
	Rat died at birth of litter					
Rat 4	1641	8	5·6	1	45	Bread and milk
	1715	3+3 dead	5·6	3	29·6	,,
	1788	4	7	0	—	,,
	Died with a cold					
Rat 5	1616	5	6·8	5	34·4	,,
	1677	3+2 dead	4·66	3	42·6	,,
	1746	8	6	6	40	,,
	Died at birth of litter					

The results may be summed up as follows:

1. The litters were of small size, 8 being the greatest number born, while the stock animals have, on an average, 9 or 10 young per litter and have had as many as 17.

2. Many of the young were below average weight at birth (5·79 g. is the average weight for this stock of rats).

3. There was noticeable failure to rear all 6 young, even when bread and milk were given to the doe after the birth of the litter. This fact, together with the low weight at birth, suggests that the oatmeal diet is unsatisfactory for gestation.

4. When the oatmeal diet was continued during lactation there was not only a failure in rearing the young, but any of them which survived weighed only about 20 g. instead of approximately 40 g. at weaning time. The young rats had very poor and thin fur.

Thus the oatmeal diet is not satisfactory during lactation or gestation, but, as far as this experiment is concerned, the lactation test is hardly a fair one, because the young were often of low weight and so might be weakly from birth. Further experiments therefore were made in which the does were given the experimental diet only after the birth of the litter.

Exp. 2. The three following diets were given and at least three rats were fed on each diet.

(*a*) Raw oatmeal and water.

(*b*) Cooked oatmeal and water.

(*c*) Cooked oatmeal, butter, salt mixture and water as given in Exp. 1.

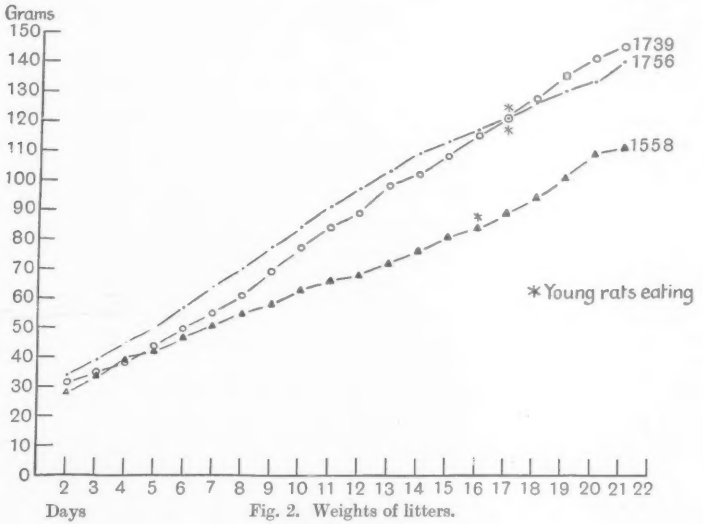

Fig. 2. Weights of litters.

Mothers' diets.

○——○ 1739 Cooked oatmeal and water.
·——· 1756 Raw oatmeal and water.
▲——▲ 1558 Cooked oatmeal, butter and salt mixture.

Representative curves of the growth of the litters are shown in Fig. 2. The litters seem to grow equally if the mother receives either cooked or raw oatmeal and to do less well when she is given the diet of oatmeal, butter and salt mixture. From results obtained with other unpublished work it is possible that this failure is due to the food containing too great a proportion of vitamins A or D (or both) as compared with vitamin B. The oatmeal, butter and salt mixture diet is low in vitamin B content for the lactating rat, because

symptoms due to this lack appeared in some of the young. It was thought that the low percentage of protein in the diet might account for the poor weight of the young at weaning, and therefore an attempt was made to supplement the oatmeal with other proteins. Butter and salt mixture were omitted.

Exp. 3.

(*a*) Diet of 20 g. raw oatmeal, 1 g. gluten and 20 cc. water.

(*b*) Diet of 20 g. raw oatmeal, 1 g. gelatin and 20 cc. water.

(*c*) Diet of 20 g. raw oatmeal, 1 g. egg albumin and 20 cc. water.

(*d*) Diet of 20 g. raw oatmeal, 1 g. food casein and 20 cc. water.

(*e*) Diet of 20 g. raw oatmeal, 1 g. purified caseinogen and 20 cc. water.

As in Exp. 2 at least three rats were fed on each diet.

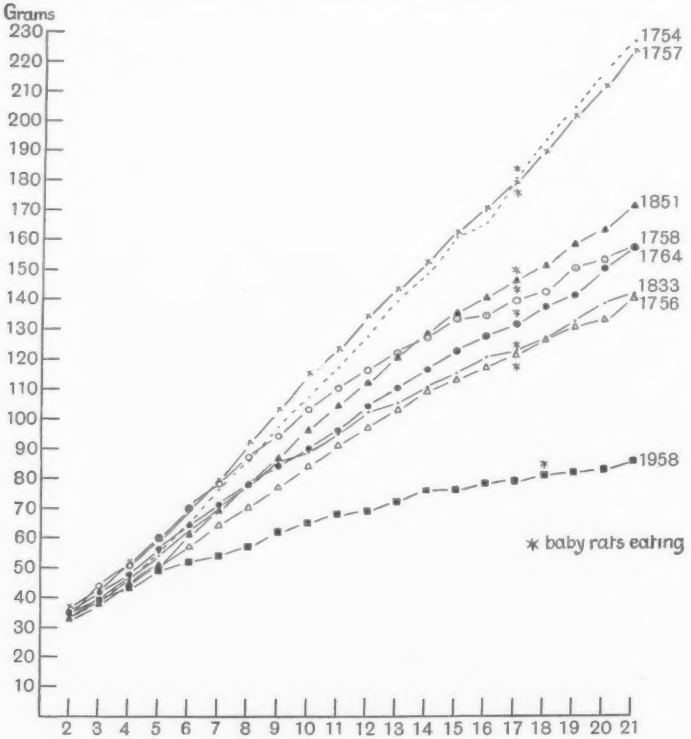

Fig. 3. Weights of litters.

Mothers' diets.

△——△ 1756 20 g. raw oatmeal + 20 cc. water.
×——× 1757 „ „ + 1 g. food casein + 20 cc. water.
▲——▲ 1851 „ „ + 1 g. egg albumin + „
○——○ 1758 „ „ + 1 g. gluten + „
●——● 1764 „ „ + 1 g. gelatin + „
•——• 1833 „ „ + 1 g. pure caseinogen + „
■——■ 1958 Synthetic diet containing 10 % of pure caseinogen.
•----• 1754 Bread and milk (control).

One curve from each experiment is given in Fig. 3, and it is obvious that the addition of other protein to the mother rat's diet produces better growth in the young.

Conclusions.

From the experiments described in this paper, it is seen that oatmeal used as the sole source of protein in the diet provides for growth in the rat, but the rate of growth is slower than when the animals are given a mixed diet such as kitchen scraps and bread and milk. Litters were born, which shows that the oatmeal diet was also adequate for fertilisation. From the point of view of gestation and lactation, however, the diet was by no means satisfactory. It is probable that the quantity rather than the quality of the oat protein is responsible for this failure. Had the quality of the protein been poor, it is unlikely that the animals themselves would have attained such good weight.

The protein in the given diet would be about 10 %. Even with caseinogen (which is regarded by nutrition workers as a good protein) the author has found very slow growth for the suckling rats when the doe was fed on a synthetic diet containing only 10 % of this protein (see Fig. 3). In fact when the mother rat had the oatmeal diet the growth of the young was distinctly better than when she was given a synthetic diet containing 10 % of caseinogen.

Except pure caseinogen, all the proteins added to the oatmeal diet of the mother produced better growth in the suckling rats. Even gelatin, which is regarded as a poor protein, and is lacking in the essential amino-acids tryptophan and cystine, caused some improvement in the growth of the young. This also seems to support the suggestion that oatmeal proteins are of good quality, but the amount present is too low for a good diet for a lactating animal. Osborne and Mendel [1918] found that both caseinogen and gelatin were effective supplements for oat proteins, but they were dealing with direct growth, and not with mammary secretion, consequently their results are not strictly comparable with those discussed here. According to Wiley [1917] the oat protein soluble in alkali constitutes 11·25 % of the whole grain, while the amounts for the other two proteins are 1·25 % and 1·5 % respectively. If the "oat casein" is similar to milk caseinogen in composition, the above figure suggests an explanation as to why the addition of pure caseinogen to the oatmeal diet gave less good results than the addition of other proteins.

The great improvement due to the supplementing effect of food casein [Hartwell, 1922] is worthy of comment. Simple experiments show that this substance contains two proteins, probably lactalbumin and caseinogen, and since the addition of egg albumin gave better results than gelatin, gluten or pure caseinogen, it is reasonable to assume that the lactalbumin is largely responsible for the noticeable improvement in Curve 1757.

Not only did the extra protein result in better growth of the young rats, but also in an improvement in general condition. Their fur was thick and, even when the young were not up to standard weight, they were quite normal as regards general condition.

SUMMARY.

1. The proteins of oatmeal are of good value as regards growth in rats.

2. For gestation and lactation a diet of oatmeal, butter and salt mixture is not adequate. This is probably due to the quantity rather than the quality of the protein.

3. The addition of food casein, gluten, gelatin and egg albumin to the oatmeal diet produced better growth in the suckling young.

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XCVIII. THE HEAT COAGULATION OF EGG-ALBUMIN.

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It has recently been suggested by Wu and Wu [1925] that tyrosine is split off when egg-albumin is coagulated by heating. This observation seemed quite probable since it is known that tyrosine is very readily eliminated from proteins. For instance, Fürth and Fischer [1924] found that tyrosine was among the first products of the tryptic digestion of caseinogen and fibrin. As the experiments of Wu and Wu were not decisive it was resolved to repeat and amplify them.

The Weiss [1919] modification of the Millon reaction seemed to offer the most specific and delicate independent test for tyrosine and could be used to detect 1 part of tyrosine in 500,000 parts of solution. It was also decided to coagulate the albumin solutions only partially in order to avoid, as far as possible, the danger of secondary hydrolysis. The total nitrogen determinations were carried out by Pregl's [1924] modification of Parnas and Wagner's micro-Kjeldahl method which will measure 0·02 mg. of nitrogen with certainty.

The crystalline egg-albumin was prepared from fresh hen's eggs in the usual way and recrystallised twice: it was then dialysed in presence of a little thymol until free from ammonium sulphate. A minimal amount of acetic acid was added near the end to aid the removal of ammonia.

2 cc. of albumin solution containing 4·9 mg. of nitrogen per cc. were diluted with 10 cc. of water and heated for 15 to 30 minutes at 60° or 70° and the protein matter precipitated by 15 cc. of diluted "dialysed iron" solution and a trace of potassium sulphate. 5 cc. of the filtrate were used for the nitrogen determination and 3 cc. for the tyrosine test.

Although it should have been possible to detect, by the Weiss reagent, one-tenth of the tyrosine present in the albumin if it had been split off on coagulation, no trace of it could be found. Further, the amounts of total nitrogen found in the filtrate were very small and constant within the limits of experimental error. They were of the order of 0·5 % of the nitrogen used and were almost certainly due to traces of ammonia from the ammonium sulphate used in the preparation of the albumin. They tended to decrease with the longer times of heating as if the substance were adsorbed on the

coagulum. The total nitrogen determinations are not sufficiently delicate to prove the absence of tyrosine but they do show that there is no general breakdown of the molecule under the conditions of the experiments. Exactly similar results were obtained when gradually increasing amounts of (1) acetic acid, (2) caustic soda, were added until there was no coagulum on heating.

As these results were directly opposed to those of Wu and Wu, their experiments were repeated using a thymol-free solution, since the Folin reagent gives a strongly positive reaction with thymol. 20 cc. of 1 % albumin solution and 25 cc. of water were heated in boiling water for 15 minutes, cooled and precipitated with 3 cc. of $2N/3$ sulphuric acid and 3 cc. of 10 % sodium tungstate solution. 0·25 cc. of the Folin and Denis [1912] phenol reagent and 3 cc. of 20 % sodium carbonate solution were added to 35 cc. of the filtrate and colour comparisons made with a 1/20,000 tyrosine solution. The experimental values are expressed as mg. of apparent tyrosine per g. of albumin.

	Nitrogen % total used	"Tyrosine" F and D	Increase "Tyrosine"	Tyrosine Weiss
Unheated	0·96	6·1	—	—
15 mins. 100°	0·71	5·6	−0·5	−ve
Unheated	—	8·6	—	—
15 mins. 100°	—	6·8	−1·8	−ve
15 mins. 70°	—	7·7	−0·9	—
15 mins. 60°	—	7·0	−1·6	—
Unheated	—	8·2	—	—
15 mins. 100°	—	7·2	−1·0	−ve
15 mins. 100°	—	8·2	0	−ve

The Molisch carbohydrate test was also negative.

These results are entirely contrary to those of Wu and Wu, in that a decrease in the colour of the phenol reagent is observed in all cases. This decrease is probably due to adsorption of the colour-producing substance on the coagulum.

If the colour was due to tyrosine, the Weiss-Millon reagent should have given a strong positive result. The absence of any colour with this reagent proves that tyrosine is not liberated from albumin on coagulation. Further, the nitrogen determinations show that no measurable amount of nitrogenous substance is liberated at all. If half a per cent. of the total nitrogen in the albumin had been split off it would be detectable by the micro-Kjeldahl method used.

It was possible that any tyrosine liberated would be adsorbed on the coagulum or protein precipitate, so 5 cc. of 1/10,000 standard tyrosine solution were added to 10 cc. of 1 % albumin and 10 cc. of water and the determinations repeated.

	"Tyrosine" F and D	Increase "Tyrosine"
Tyrosine added	5·0	—
Unheated	6·25	—
15 mins. 70°	6·95	+0·70
15 mins. 100°	7·05	+0·80

This clearly indicates that tyrosine is not adsorbed on the coagulum since with increasing time of heating and corresponding increase in the amount of coagulum the tyrosine found does not decrease. The figures obtained are practically identical with those without any addition of tyrosine so that the effect of further additions was examined, the solutions being heated in boiling water for 15 minutes.

5 mg. of tyrosine added	7·25 mg. "tyrosine" found
10 " "	15·0 " "
15 " "	21·5 " "

In all these cases tyrosine can easily be detected by the Weiss test. The first 5 mg. or so of tyrosine added have some action on the chromogenic substance already present but the nature of this action has not been determined.

The nitroprusside tests, as modified by Walker [1925], gave an immediate coloration with any of the filtrates and it was shown that, while cystine gave no colour with the Folin and Denis reagent, an intense blue colour was given by cysteine. It is accordingly suggested that some substance containing a thiol group is liberated when albumin is precipitated by means of sodium tungstate or ferric hydroxide and that this sulphur-containing substance reduces the phenol reagent. It was shown that potassium permanganate was decolorised by the filtrate.

It cannot be too strongly emphasised that the delicate phenol reagent of Folin and Denis may only be used to estimate tyrosine in the proved absence of all readily oxidisable substances, as any such substance will produce the blue colour [cf. Gortner and Holm, 1920].

It does not necessarily follow that the production of this reducing substance involves any chemical decomposition in the present case as it may well be due to desorption accompanying precipitation.

It may be noted that fresh egg-white does contain free tyrosine which collects at the anode in large quantities when an egg-white solution is electrolysed. It may also be detected in the mother liquors after precipitating the crystalline egg-albumin. Tyrosine may thus be found in a badly prepared specimen of albumin. A normal albumin solution which has just commenced to become foul will also contain free tyrosine and the amount of this free tyrosine undoubtedly does increase when the solution is heated.

Sörensen and Sörensen [1925] have also come to the conclusion that heat coagulation is unaccompanied by decomposition but it has not been possible to consult the original paper.

Summary.

1. No evidence can be obtained that tyrosine is split off when albumin is coagulated by heating.

2. A reducing substance is present in the filtrate when an albumin solution is precipitated by sodium tungstate or colloidal ferric hydroxide. This substance probably contains a free —SH group.

3. The Folin and Denis phenol reagent cannot be used to estimate tyrosine in the presence of easily oxidisable substances.

The authors wish to thank Professor S. B. Schryver for his constant interest and advice during the course of the work.

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XCIX. THE PREPARATION AND SOME PROPERTIES OF PURIFIED DIPHTHERIA TOXOID.

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The desirability of purifying toxins and antigens in general need not be emphasised. One of the chief difficulties experienced in the employment of the Schick test for susceptibility to diphtheria is the presence in the toxic filtrates of some substance which produces the "pseudo" reaction and hinders the correct interpretation of the specific effect due to the toxin. A similar reacting substance appears to exist in the filtrates of the streptococcus which have recently been used in the Dick test for susceptibility to scarlet fever [Dick and Dick, 1924]. In both cases the substance causing the "pseudo" reaction is definitely more heat-stable than the specific substance, but beyond this fact nothing is known of its constitution or properties. Further, many culture filtrates to a greater or less degree contain substances which tend to interfere with the general condition of the animal being immunised [Watson and Langstaff, 1926]. These substances may cause temperatures and other non-specific reactions which preclude the injection without injury of the increasingly large doses of toxin necessary for the successful production of antitoxin. The presence of similar non-specific substances has recently been suspected in the culture filtrates of *B. Welchii* and *B. coli* by Dalling and Mason [1926]. The production of immunity to lamb dysentery was interfered with and in some cases lameness and even death caused by some component of the culture filtrates which formed the basis of their prophylactic mixtures. Similar difficulties exist in the clinical aspects of the problem. The active immunisation of children and adults against diphtheria and scarlet fever is attended in a small percentage of instances by painful reactions which seem to be due to some non-specific constituents of the toxin-antitoxin mixtures at present in use and not to the active principle itself. The clinical use of culture filtrates treated with formaldehyde has been explored by Park and his colleagues [1924] in America. They found that such toxoid preparations were valuable antigens [see also Glenny and Hopkins, 1923] but concluded that the toxoid preparations which had no toxicity in guinea-pigs could not be properly used in human beings in doses larger than 0·5 cc. because of the marked "pseudo" reaction. With some of the later toxoid preparations, these workers find that the difficulties connected with the local reactions have been

partially overcome. Larson and Eder [1926] also support the use of simplified immunising preparations and claim that toxic culture filtrates which have been detoxified by sodium ricinoleate are fully equal, and in many respects superior, to toxin-antitoxin mixtures as immunising agents.

There would seem to be two urgent problems awaiting solution in this field of immuno-chemical research. The first problem is to convince ourselves that under no circumstances are the toxin-toxoid reactions reversible and to determine the relative values of such detoxifying agents as formaldehyde and the salts of ricinoleic acid, etc. The work of Glenny and his colleagues in England, Ramon in France, and Park and his co-workers in America have failed up to the present to expose any sign of such a reversal in the case of culture filtrates detoxified with formaldehyde but information on the nature of the action of the formaldehyde is lacking. Our knowledge of the action of salts of ricinoleic acid on toxin is confined to the work of Larson and his co-workers and no extended use of their toxoids as immunising agents has yet been possible. The second problem awaiting solution is to purify these toxoid antigens. The active principle of culture filtrates is probably confined to a very small fraction of the total material and as shown later, it is possibly associated with less than 1 % of the nitrogenous material in the culture filtrates. If this is so, the purification of the antigens should do much to eliminate the non-specific reactions which as Park and his colleagues state preclude the proper use of atoxic preparations for the immunisation of human beings. Further, investigation of the constitution of antigens and the chemical processes of immunity may be facilitated if comparatively pure antigens are substituted for the highly impure culture filtrates which at present form the basis of our attack on the various problems.

Research in this field has been facilitated during the past few years by the work of Ramon [1922], whose flocculation test for assaying the strength of diphtheria toxin and antitoxin has received almost universal recognition. The present work is essentially a development of that of Watson and Wallace [1924, 1, 2] on the purification of culture filtrates. Some of the properties of purified toxin were described but at the time the only well-established methods of testing the various fractions were by the use of animals, and progress was necessarily slow. This work has recently been confirmed by Moloney and Weld in Canada [1925]. The present paper consists of a brief survey of the method of purification of culture filtrates containing toxins partially or completely converted into toxoids by formalin together with a discussion of some of the properties of the purified substance. The technique of Ramon was employed for testing the value of the fractions and has thrown light on many of the facts which were described in the previous paper [Watson and Wallace, 1924, 2] and for which at the time no explanation was forthcoming.

Terminology.

A survey of the terminology at present in use in the field of immunological research has recently been made by Glenny, Pope and Waddington [1925]. The term *purified toxin* is used in the present paper to denote the active fraction obtained from culture filtrates of the diphtheria bacillus by precipitation at low temperature with glacial acetic acid and rapid separation of the precipitate, which is then dissolved in cooled dilute caustic soda to p_H 8·0 [Watson and Wallace, 1924, 2]. *Purified toxoid* is employed to denote the active fraction obtained from diphtheria toxoid by precipitation at 35° or less with glacial acetic acid and separation of the precipitate which is then dissolved in dilute caustic soda to p_H 8·0. Many of the purified toxoid solutions used during the course of the work were not completely atoxic, the original toxins having been only partially converted into toxoids before concentration. The terms *ultra-purified toxin and toxoid* are employed to denote purified toxin or toxoid which has been subjected to further purification by means of acetic acid precipitation, or alcohol precipitation [Moloney and Weld, 1925] or by dialysis against water for periods of 48–72 hours.

Methods.

The media used for culture filtrates were prepared by different variations of enzyme digests of horse muscle [Watson and Langstaff, 1926] or by the solution of commercial peptones in muscle extracts. The media, sterilised by filtration through a Seitz filter press followed by a short steaming, were inoculated with Park William No. 8 strain of *C. diphtheriae* and incubated for 10 days in the case of digest medium and 7 days in the case of peptone medium. To obtain diphtheria toxin, the filtrates from each culture were then tested and graded accordingly. Each group of cultures was filtered through a Berkefeld candle either without preservative or else with 0·5 % added phenol or toluene. To prepare diphtheria toxoid a similar technique was adopted but the cultures were treated directly with 0·5 % neutralised formalin. The filtrates were either concentrated at this stage or else were incubated for varying periods (sometimes with the addition of extra formaldehyde) to complete the toxin-toxoid change. A preliminary assay of a group of bulked and filtered toxoids was carried out. Total, Van Slyke, and precipitable nitrogen [Watson and Wallace, 1924, 1] and total solids content were estimated. The Lf value[1] was also determined in each case. A similar series of data was accumulated for the purified solutions and from these observations some idea could be obtained as to the degree of purity that had been reached relative to the original unpurified toxoid.

[1] The Lf dose is that amount of "toxin" which is equivalent to one unit of antitoxin by the flocculation test [Glenny and Okell, 1924].

The action of acids and alkalis on formalised culture filtrates of *C. diphtheriae*.

The action of a strong acid, *e.g.* hydrochloric acid and a strong base, *e.g.* sodium hydroxide on a typical formalised culture filtrate is shown in Fig. 1 (Curve A).

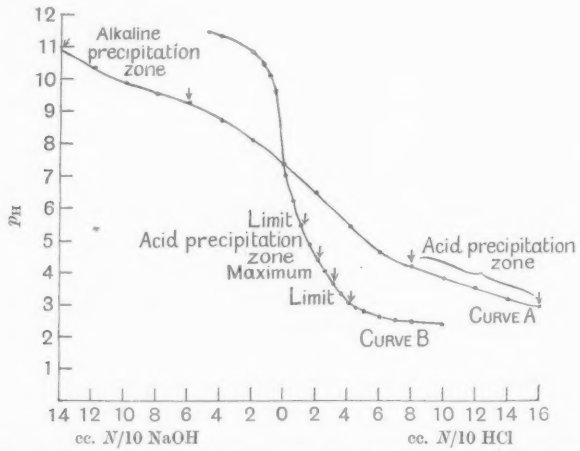

Fig. 1. Buffer curves of diphtheria toxoid and purified diphtheria toxoid.

Curve A. Diphtheria toxoid. Curve B. Purified diphtheria toxoid.

Varying amounts of decinormal acid and alkali were added to the filtrate and the p_H of the various samples estimated electrometrically[1].

By plotting the amounts of acid or alkali added against the corresponding p_H of the solution we obtain the "buffer" curve of the filtrate. The curve obtained in this case is typical for culture filtrates prepared from medium made by the prolonged tryptic digestion of horse muscle [Watson and Langstaff, 1926]. There are two well-marked precipitation zones, (*a*) an alkaline zone extending from p_H 9·25 to 11·0, and (*b*) an acid zone extending from p_H 2·9 to 4·2. The alkaline precipitate consists mainly of phosphates while the acid precipitate is composed of material of high molecular weight which experimental work has shown contains a proportion of the active principle of the filtrates. The action of other acids is similar to that of hydrochloric acid but the amounts of acid required to reach the precipitation zone depend, of course, on the degree of dissociation of the acid. Acetic acid is the most useful from many points of view. To obtain the most active fractions each batch of filtrates should be subjected to a separate study and the acid precipitation zone carefully explored. The technical details of such studies and their application to the "working up" of filtrates to the maximum advantage

[1] We are indebted to our colleague Mr Pope for the electrometric determinations of p_H carried out during the course of the work.

will be dealt with in a future publication. For the present work, however, 1 % glacial acetic acid has been used as the precipitating agent and the precipitate so obtained subjected to a systematic chemical study. It is of interest here to include data on the amount of precipitate produced by the action of 1 % glacial acetic acid on various types of culture filtrate. The presence of phenol or formaldehyde plays little, if any, part in determining the amount of precipitate formed. Table I gives the results obtained. For the estimations, 300 cc. of the culture filtrate were precipitated with 1 % glacial acetic acid and the precipitate coagulated by heating in a water-bath to 100° for 10 minutes. The solution was then cooled to room temperature and filtered. The precipitate was washed with 300 cc. of distilled water, transferred to a Kjeldahl flask and the nitrogen estimated in the usual way.

Table I. *Nitrogen precipitable by* 1 % *glacial acetic acid from various types of culture filtrates.*

Type	No.	Total nitrogen mg./100 cc.	Precipitable nitrogen mg./1000 cc.	Percentage	
				Precipitable nitrogen	Non-precipitable nitrogen
Peptone X + horse muscle extract	J 3543	442·4	21·7	0·5	99·50
Peptone Y + horse muscle extract	J 3558	281·4	50·8	1·82	98·18
Peptone Z + horse muscle extract	J 3541	320·0	44·5	1·4	98·60
Tryptic digest (*a*) 3·5 hrs. at 40°.					
Horse muscle (Watson and Wallace)	J 3550	439·6	42·5	0·97	99·03
	J 3554	436·8	40·9	0·94	99·06
(*b*) 2·5 hrs. at 37°.					
(Watson and Langstaff)	TA 20	250·5	30·27	1·21	98·79
	TA 21	245·0	11·93	0·49	99·51
(*c*) 72 hrs. at 15°.					
(Watson and Langstaff)	TME 99	180·6	33·6	1·86	98·14
	TME 100	277·4	11·7	0·42	99·58

In all cases the amount of nitrogenous material removed from the filtrate is very small and it would appear that the active principle, whatever its nature, must form only a small percentage of the total material of the culture filtrate.

The general technique for preparing purified toxoid.

The method is essentially that of Watson and Wallace [1924, 2] and consists of the acetic acid precipitation of the culture filtrates of *C. diphtheriae* which have been treated with formalin. If completely atoxic solutions are required the formalised filtrates are incubated until the M.R.D. [Glenny and Allen, 1921] of the filtrates is negligible. The distribution of the active principle between the precipitate and supernatant fluid varies with the type of filtrate used and also with the presence of preservatives or other substances which may affect the stability of the toxoid molecule. The details of the large scale methods for precipitating culture filtrates and the working up of "super-

natants" to the best advantage will be dealt with in a future publication but the following brief outline of the process may be included here.

Formation and separation of the precipitate. The filtrates are placed in double Winchester quart bottles, 4 litres per bottle, and 1 % glacial acetic acid is added. The precipitate produced is allowed to settle out and is then removed by centrifugation. When small quantities of filtrate are being handled, this may be done in glass pots in a large centrifuge. After the preliminary centrifugation the supernatant is carefully siphoned off and replaced by an equal quantity of 0·7 % saline or sodium acetate-acetic acid mixture adjusted to the p_H of the acid precipitation point (*ca.* 3·8). If distilled water or saline of p_H above or below this point is used appreciable losses may take place during the washing. One washing is usually sufficient, but if a more complete removal of pigment from the precipitate is required one or two more washings will effect this. If large volumes of toxoid are being employed the Sharples centrifuge offers many advantages, and the removal of the precipitate may be carried out almost quantitatively if the interior of the bowl is lined with a sheet of celluloid. One drawback with this type of centrifuge is that the final washing of the precipitate cannot be carried out as satisfactorily as with a large centrifuge having glass pots. From every other point of view, however, the Sharples machine is to be preferred for the large scale concentrations.

Removal and solution of the precipitate. The celluloid cylinder on which the precipitate collects is removed from the metal bowl of the centrifuge with forceps and the precipitate scraped into glass mortars and dissolved in physiological saline or distilled water buffered to p_H 8·0. Precautions to keep the solution under ice-cold conditions such as are necessary in the production of purified toxin would appear to be unnecessary in the preparation of purified toxoid from most culture filtrates.

Chemical studies of these purified antigens can be carried out more conveniently if no preservatives are present. Phenol especially, as will be shown later, interferes with the stability and chemical properties of the solutions.

Factors influencing the preparation of purified toxoid. It was shown in the previous communication that the amount of acid used, the temperature of precipitation and speed of solution of the precipitate, all played an important part in determining the value of purified toxins. Purified toxoid is a much more stable substance. For most toxoids the temperature of precipitation (provided it is less than 37°), and the speed with which the precipitate is brought into solution, play only a small part in determining the strength of the purified solution as judged by Lf measurements. The amount of acid added still remains an important factor. As a general rule, the smallest amount of acid consistent with the production of an appreciable precipitate seems to give the best results. This amount of acid should be found experimentally for each batch of filtrates.

A typical concentration of diphtheria toxoid. Table II shows the results of

a typical concentration. Forty-eight litres of a toxoid containing 8·5 Lf units per cc. were precipitated with 1 % glacial acetic acid in 12-litre batches. The precipitated toxoid was spun in the Sharples centrifuge and the precipitates dissolved in 1500, 750, 375 and 187·5 cc. of saline respectively, the p_H adjusted to 8·0 and the solutions filtered through a Berkefeld candle. The total nitrogen and Lf values of the unfiltered samples and the total solids content and Lf value of the filtered samples were determined.

Table II. *The preparation of purified toxoid using the Sharples centrifuge for separation of precipitate.*

	Purified toxoid			
	Lf units per 10 cc.	TF 45°* (min.)	Total N mg./100 cc.	Lf units per 100 mg. N
CT 15 A	200	105	12·5	16,000
CT 15 B	460	90	21·3	21,590
CT 15 C	1040	8	60·3	17,250
CT 15 D	1800	3	90·3	19,930
	Filtered purified toxoid			
	Lf units per 10 cc.	TF 45°* (min.)	Dry weight mg./10 cc.	Total solid per Lf unit (mg.)
CT 15 A	200	65	125·5	0·627
CT 15 B	360	40	116·0	0·322
CT 15 C	720	3	120·5	0·167
CT 15 D	1300	10–8 at R.T.	102·0	0·078

* TF 45° = flocculation time at 45°.

Several facts are seen from the table. These precipitates may apparently be dissolved to give almost any required concentration of Lf units, the volume of the purified solution being limited only by the solubility of the material. The greater the strength of the solution the more rapid is the flocculation with antitoxin. The strongest sample, for instance, flocculated in a few minutes with almost an equal volume of serum containing 200 units of antitoxin per cc. If the antigenic value of a solution depended on the strength of the toxoid and the rapidity of its flocculation with antitoxin alone (which is unlikely) these purified solutions should be ideal antigens. Apart from this aspect of the problem—which is, of course, the most important one—these solutions offer us a more satisfactory starting point for researches on the chemical constitution of antigens and the part they play in immunity processes than the culture filtrates of the bacillus. The total solids content of the most potent filtered purified toxoid of the series was only 0·078 mg. per Lf unit, and when it is remembered that a considerable percentage of this was added salt the very small proportion of a culture filtrate which is occupied by the active fraction will be realised. The active principle—whatever its nature—is adsorbed to some extent by kieselguhr as will be seen by the differences in value of the solutions before and after they have been filtered through a Berkefeld candle. This adsorption increases with the amount of precipitate in solution. Once a purified solution is produced it can be diluted to any required Lf value with saline or other purified toxoid of known Lf value.

THE ULTRA-PURIFICATION OF PURIFIED SOLUTIONS.

The active fraction precipitated from the culture filtrates can be purified still further by such methods as reprecipitation with a small amount of acetic acid, or with alcohol [Moloney and Weld, 1925], or by the dialysis of the solutions in a collodion membrane against water. The degree of purity produced by any of these methods depends on the nature of the active fraction and the type of filtrate from which it was produced.

The estimation of the degree of purity of the solutions. As a simple estimate of the degree of purity attained during the stages of the concentration process the total solids and nitrogen content of one Lf unit were calculated for each of the unpurified toxoids and the various purified solutions. The ratio of the figure obtained for the purified toxoid to that for the unpurified solution gives the purity of the solution relative to the original material. The total solids concentration figures are, of course, dependent on the nature of the solvent used for the precipitate and the amounts of acid and alkali used for the p_H adjustment. The nitrogen concentration figures provide the more true estimate of the degree of purity reached. This degree of purity varies over a wide range. The active fractions may contain from 30 to 130 times less nitrogen than the original toxoid. Some of these purification figures are collected in Table III.

Table III. *The concentration of the active principle, as shown by the dry weight and total nitrogen ratios of one Lf unit.*

	No.	Total solids mg. per cc.	Total N mg. per cc.	Lf units per cc.	"Weight" of one Lf unit mg.	Nitrogen content of one Lf unit mg.	"Weight" ratio	Nitrogen ratio
Parent toxoid	TME 99	24·75	1·806	8·5	2·91	0·212	—	—
Purified toxoid	CT 15 A*	12·5	0·125	20	0·625	0·00625	4·65	33·9
	CT 15 B*	11·6	0·213	36	0·322	0·00592	9·04	35·9
	CT 15 C*	12·05	0·603	72	0·167	0·00837	17·42	25·4
Parent toxoid	TME 100	29·00	2·774	12	2·42	0·231	—	—
Purified toxoid	C4/21	1·75	—	40	0·0437	—	55·38	—
	C4/28*	0·6	—	26	0·023	—	105·22	—
	C4/34 B*	0·75	—	14	0·054	—	44·82	—
Parent toxoid	JM 3801	27·20	2·536	5·5	4·94	0·461	—	—
Purified toxoid	CT 16 A	—	0·175	36	—	0·00486	—	94·8
	CT 16 B	—	0·434	78	—	0·00556	—	82·9
	CT 16 C	—	0·895	165	—	0·00543	—	84·9
Parent toxoid	BJ 3766	—	2·755	4·5	—	0·612	—	—
Purified toxoid	CT 17 A	—	0·218	44	—	0·00495	—	123·64

* Precipitate dissolved in distilled water.

No extended investigation has been carried out on the dialysis of purified toxoid but it may be of interest to include here data with regard to the dialysis of some purified solutions obtained from phenolised culture filtrates. The purified solutions can usually be dialysed for 48–72 hours against water in a collodion membrane without loss of active principle. Table IV shows the increase in the concentration of Lf units per mg. of nitrogen in the ultra-purified solution as the dialysis proceeds.

Table IV. *The increase of purity of two reconstructed antigens as dialysis proceeds.*

Period of dialysis (hours)	Concentration of Lf units per mg. N: Filtrates prepared from tryptic digest media. CJ 24 A	Filtrates prepared from peptone media. CJ 26 B
0	81·4	146
24	112·0	161
48	141·0	182
72	146·0	194

By these methods of purification, therefore, solutions which are over 100 times as "pure" as the original culture filtrates may be obtained. It is interesting to note that Hartley [1926] working with the washed precipitates from toxin-antitoxin mixtures has recently prepared active antigens which contained less than 0·002 of the nitrogen of the mixtures from which they were derived.

THE STABILITY AND CHEMICAL PROPERTIES OF PURIFIED TOXOID.

Except where otherwise stated, the material used in the following experimental work was prepared by precipitation of diphtheria toxoid with glacial acetic acid, centrifugation of the solutions in the large centrifuge and careful washing of the precipitate before solution in dilute caustic soda to p_H 8·0. No preservative was used for the purified solutions unless otherwise stated.

The stability of purified toxoid at various temperatures.

(*a*) *Temperatures below freezing point.* The effect of freezing purified toxoid in the presence of phenol (0·5 %), formalin (0·5 %), hydrogen peroxide (0·5 %) and without preservative is shown in Table V.

Table V. *The effect of freezing purified toxoid with and without antiseptic.* (TME 100. C 4/19.)

Solution	p_H	Preservative	Lf units per cc.: Before freezing	After freezing for: 3 hours	20 hours
Original toxoid (TME 100)	7·2	Formalin	12	12	12
Purified toxoid	8·0	—	12	12	12
,,	8·0	0·5 % phenol	12	8	3
,,	8·0	0·5 % hydrogen peroxide	12	12	12
,,	8·0	0·1 % formalin	12	12	No flocc.

No destruction was observed after freezing the original toxoid for 3 or 20 hours. The purified toxoid, whether unpreserved or preserved with formalin or hydrogen peroxide, suffered no ill effects from short or prolonged freezing. 75 % of the flocculating value was removed by freezing the purified toxoid when preserved with phenol. The deleterious effect of the presence of phenol as a preservative for culture filtrates was pointed out by Moloney and Weld

[1925] and Glenny, Pope, Waddington and Wallace [1925] demonstrated the ill effects of freezing toxin-antitoxin mixtures in the presence of phenol.

(*b*) *Temperatures* 5°–37°. The stability of purified toxin within this temperature range was dealt with in a previous paper [Watson and Wallace, 1924, 2], where it was shown that deterioration or modification proceeded rapidly at 37° and definitely but more slowly at 17° and 5°. After some time at the latter temperatures a state of comparative equilibrium was set up. The values of the various samples after different time periods were estimated by animal tests (Lr/500 skin measurements). The stability of purified toxoid as measured by Lf tests is well marked. Without preservative it may be kept for long periods of time without loss of flocculating power with antitoxin. The presence of phenol tends to definite destruction of the flocculating value on standing. If formalin is added to these purified toxoids, even in minute amounts, flocculation with antitoxin may be difficult to detect or the time of flocculation is increased. These facts are illustrated in Table VI.

Table VI. *Stability of purified toxoid with and without preservative at various temperatures.*

Lf units per cc.

Period in days	No preservative			Phenol 0·5 %			Formalin 0·1 %			Hydrogen peroxide 0·5 %		
	4°	17°	37°	4°	17°	37°	4°	17°	37°	4°	17°	37°
0	16	16	16	14	14	14	14	14	14	14	14	14
1	16	16	16	14	14	12–14	12	12	12	14	14	14
2	16	16	16	—	—	—	(Time of flocculation 6 days)			—	—	—
4	16	16	16	—	—	—				—	—	—
7	—	—	—*	14	14	10				14	14	14
11	16	16	10	—	—	—				—	—	—
15	—	—	—	14	14	10				14	14	14
18	16	16	—	—	—	—				—	—	—
23	—	—	—	14	14	10				14	14	14
26	16	16	—	—	—	—				—	—	—*
30	16	16	—	14	14	8				14	14	10
44	16	16	—	14	14	8				16	16	10
65	16	16	—	14	14	—				16	16	—

* Contaminated.

Some of these purified solutions have been kept for over 2 years at cold room temperature and in some cases they still retain their high flocculating power. It would seem that the "deterioration or modification" of the purified toxins described in the previous paper may have been one of modification only. The purified toxic component of the solution apparently slowly changes into the toxoid component, the reaction being facilitated by warmth.

(*c*) *Temperatures above* 37°. The behaviour of the purified toxoid without antiseptic when heated to temperatures beyond 37° is shown in Fig. 2. About 200 cc. of purified toxoids of different initial flocculating values were placed in flasks on an electric heater and taken slowly to 100° with constant shaking in the presence of glass beads. Samples were removed at various temperatures and examined for p_H and Lf values. The p_H changes of the various samples were negligible. There are indications of slight destruction before a temperature

of 100° is reached, after which complete destruction takes place very quickly. There is also some indication that the stronger toxoid is slightly more heat-labile than the weaker—a point which has been suggested by other similar experiments.

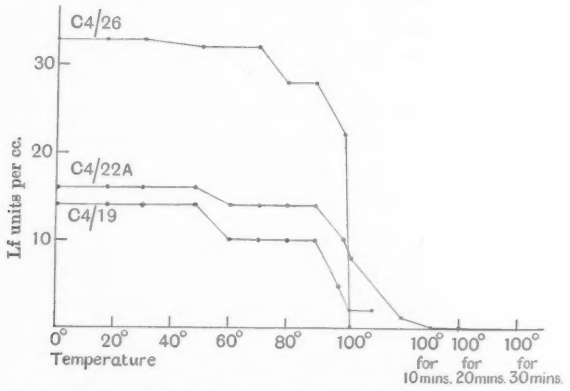

Fig. 2. The effect of heat on purified toxoids without preservatives.

The stability of purified toxoid to acids and alkalis.

Fig. 1 (Curve B) shows the buffer curve of a typical purified toxoid. The solutions were treated with $N/50$ acid and alkali. There is no alkaline precipitation zone such as exists in the case of the unpurified filtrates. The small buffer content of the solution is illustrated by the steepness of the curve. Precipitation begins at p_H 5·5 and the precipitate is redissolved at p_H 2·8. Maximum precipitation takes place between p_H 3·8–4·2. To test the stability to acids and alkalis another sample of purified toxoid (from a different parent toxoid) was adjusted to varying hydrogen ion concentrations. These samples were then, without any re-adjustment of p_H, tested for flocculating value (*a*) immediately, and (*b*) after 72 hours. The results are collected in Table VII.

Table VII. *The effect of different hydrogen ion concentrations on the Lf value of purified toxoids.*

	Tested immediately after adjustment of p_H			Tested after 3 days at 4°		
Sample	p_H	Lf units per cc.	Tf in mins.	p_H	Lf units per cc.	Tf in mins.
A	3·0	No flocc.		3·0	No flocc.	
B	4·4	Precipitation level			Precipitation level	
C	5·0	12	50	5·0	12	75
D	6·0	14	75	6·0	14	135
F	7·0	14	75	7·3*	14	105
Control	7·4	14	70	7·4	14	70
H	8·0	14	75	7·1*	14	100
K	9·0	14	135	7·8*	14	105
L	10·0	14	3 days	8·6*	14	1 day

* p_H changes due to atmospheric effect on solutions of low buffer content.

The solutions at p_H below the acid precipitation zone showed no flocculating value. At all hydrogen ion concentration levels which were definitely above the precipitation zone, however, flocculating values similar to those of the control purified toxoid were obtained. At as high a p_H as 10·0 the value remained the same, although the time of flocculation was considerably increased. This is rather a remarkable result in view of our old ideas with regard to the instability to acids and alkalis of unpurified toxin preserved with phenol. Moloney and Weld [1925] using unpurified toxoid at p_H 10·0 were unable to obtain any flocculation with antitoxins.

The stability to shaking. During the preliminary survey of the chemical properties of purified toxoid the effect of shaking on the degree and time of flocculation with antitoxin was investigated and Table VIII gives some of the results obtained.

Table VIII. *The stability of purified toxoid to mechanical shaking.*

Purified toxoid	Shaken with	Original Lf	Period of shaking (hours)	Lf after shaking
C 4/26	Glass beads	33–34	3	33–34
C 4/20	,,	7	3	5–6
C 4/22 A	,,	18	3	14–15
C 4/18	Glass beads	14	3	14 (40 secs. TF)
C 4/18	0·5 % phenol + glass beads	14	3	12 (1 day TF)
C 4/18	0·5 g. charcoal + glass beads	14	3	1 (1 day TF)

25 cc. of the purified solutions were shaken vigorously in a mechanical shaker for 3 hours with and without the addition of phenol and charcoal. To ensure uniform shaking the same number of small glass beads were placed in each bottle containing the mixtures. Shaking alone may slightly affect the flocculating value but no appreciable diminutions have been observed. Shaking with 0·5 % pure phenol produces a slight drop in the flocculating value and the time of flocculation is considerably increased. That the active principle is adsorbed by charcoal is shown by the drop in the Lf value from 14 units to 1 unit after 3 hours' shaking.

The stability to rapid aeration.

That solutions of purified toxoid are also comparatively stable to mild oxidation is shown by the fact that if a rapid current of air or oxygen is passed at room temperature through such solutions containing a small amount of capryl alcohol (to reduce frothing) for several hours, there is no loss of flocculating value. At temperatures approximating to 50° destruction is facilitated although the degree of destruction varies with purified toxoids prepared from different parent toxoids.

The chemical properties of purified toxoids.

Culture filtrates prepared from media made by the tryptic digestion of horse muscle consist of muscle extractives and breakdown products of the muscle split off by the action of the enzyme. An analysis of the nitrogenous

components of one of these culture filtrates by the method of Wasteneys and Borsook [1924] showed the following results:

	Mg./10 cc.	% total N
Total N	34·00	—
Protein N	0·07	0·021
Metaprotein N	2·28	6·7
Proteose N	11·59	34·1
Peptone N	15·2	44·7
Sub-peptone N	0·025	0·07
Residual N	4·835	14·409

A large proportion of the nitrogen is thus present as metaprotein, proteose and peptone.

During the growth of the diphtheria bacillus, various nitrogenous changes take place. There is an increase in the nitrogen precipitable by acetic acid and in the Van Slyke nitrogen [Watson and Wallace, 1924, 1]. Very little is known of the action of formalin in the production of diphtheria toxoid. There is a progressive decrease in free amino-nitrogen as the action proceeds and the toxicity of the solution to guinea-pigs steadily diminishes although the flocculating value with antitoxin remains the same. Formaldehyde can always be detected in the final atoxic antigens [Rimini-Schryver method, Schryver, 1910]. Diphtheria toxoid is, therefore, a very complex solution. Although the nitrogenous active fraction precipitable by acetic acid from the toxoid still contains a proportion of extraneous components, a survey may be included here of some of its chemical properties. After dialysis this active fraction gives the same reactions as before, but the colour tests are somewhat less marked. Table IX summarises the chief chemical reactions which survive the purification process.

Table IX. *Some chemical reactions of diphtheria toxoids.*

Test	Diphtheria toxoid	Purified toxoid
For elements		
Nitrogen	+	+
Sulphur	+	+
Phosphorus	+	*
Iron		*
For proteins		
Xanthoproteic	+	+
Hopkins-Cole	+	*
Millon	+	+
Molisch	+	*
Biuret	+	+
For Carbohydrates		
Fehling before hydrolysis	−	−
Fehling after HCl hydrolysis	−	−
For formaldehyde		
Rimini-Schryver	+	+

* In some cases negative results were obtained, in others the test was positive.

As Moloney and Weld [1925] have shown, sulphur survives this purification although whether it is an essential constituent of the toxoid molecule is not known. The fact, however, is interesting in view of Davis and Ferry's work

[1919] on the importance of the sulphur-containing amino-acid cystine for toxin production. Phosphorus apparently is not an essential constituent and the iron is probably derived wholly or in part from the centrifuge used for the concentration. It is invariably present if the Sharples machine is used for this purpose. The active fraction gives most of the usual protein reactions, although contrary to the findings of Moloney and Weld [1925] not all of our purified toxoids give a tryptophan reaction. Formaldehyde is always detectable in the purified toxoid and is, moreover, somewhat difficult to remove even by dialysis.

90 % or more of the total solids from the purified solutions are combustible. No quantitative determinations of carbon, nitrogen, etc. have yet been made. A small percentage of amino-nitrogen can always be detected in the purified solutions and 60–85 % of the total nitrogen is precipitable by Hedin's tannic acid method for the determination of proteose nitrogen.

Summary.

1. The active principle of toxic filtrates of *C. diphtheriae* is confined to a very small fraction of the filtrates.

2. By formalising the filtrates and treating them with acetic acid, a highly active fraction is precipitated.

3. This active fraction can be further purified by reprecipitation methods or by dialysis, and solutions as much as 100 times or more less impure than the original toxoid obtained.

4. Purified toxoid, like diphtheria toxoid, is a very stable substance.

5. The purified solutions are protein in nature and contain sulphur and occasionally phosphorus.

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C. SOME PHOSPHORUS COMPOUNDS OF MILK.

II. THE LIBERATION OF PHOSPHORUS FROM CASEINOGEN BY ENZYMES AND OTHER AGENTS.

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Of all the naturally-occurring proteins, caseinogen has perhaps been most studied, but there is remarkably little information available in the literature as to the mode of chemical attachment, and none as to the biological significance, of the group which gives caseinogen its place in protein classification as a member of the small group of phosphoproteins. That the phosphoric acid group in these proteins is not in the nature of an inorganic impurity, but is definitely a portion of the molecule, is now generally accepted, and rests on a secure foundation. The experiments described in this paper were undertaken with a view to eliciting further information as to the mode of combination of phosphorus in caseinogen, with an ultimate intention of throwing light on the *raison d'être* of the phosphoprotein in the natural foodstuff of the young animal. The results recorded here must be regarded as merely preliminary steps toward the solution of these problems.

With regard to the significance of the phosphoric group present in organic combination in caseinogen there are three possibilities. Either this group (*a*) has some definite part to play in the economy of the suckling, or (*b*) is there because of certain synthetic necessities of the mammary gland, or (*c*) is present adventitiously, and, in the milk, could be replaced equally well by inorganic phosphoric acid as far as the needs either of the mother or of the growing suckling are concerned. Although there is no direct evidence at present, there are nevertheless a number of reasons why the third possibility would appear unlikely. Thus the natural food of the young of all animals contains similar substances. The caseinogen of mammalian milk has its analogues in the vitellin of the egg-yolk which nourishes the growing chick, in the ichthulin which performs a similar function in the roe of fishes and in a phosphoprotein occurring in frogs' eggs.

If these compounds had no significance in virtue of their *organically* bound phosphorus, it is difficult to understand why they should be produced by organs as widely different as the mammary gland of the cow and the ovary of the herring, for the same purpose, namely, that of forming a quantitatively large part of the only foodstuff of the young animal. Did they not subserve some particular function, one would expect that the phosphorus would be supplied in inorganic form.

Assuming that the organically bound phosphorus of the food is entirely converted into inorganic phosphates either in the lumen or in the walls of the digestive tract, and is therefore absorbed as such, it would appear that any advantage derived from the organic phosphorus by the young animal is connected with the maintenance of phosphoric acid in un-ionised form for a shorter or longer period. This would allow of absorption of phosphorus at different levels in the intestine and prevent its immediate conversion (in presence of Ca ions and at the p_H of the intestinal contents) into insoluble calcium phosphate no longer capable of being taken up at all. Some experiments brought forward in this communication would indicate that, although phosphorus in organic combination is easily removed from the caseinogen molecule by digestive enzymes, the further hydrolysis to inorganic phosphate does not proceed quite so easily as might have been anticipated.

Historical.

The presence of phosphorus as an integral part of the caseinogen molecule was clearly demonstrated by Hammarsten [1872, 1877, 1883], who in 1872 proved that the phosphorus content of caseinogen, derived from the milk of any one species, and separated from all mineral impurities, was a constant quantity characteristic for the species.

Action of pepsin. Lubävin [1871] had early noticed that when gastric juice was allowed to act upon caseinogen, a greyish deposit was gradually formed during the course of the experiment and collected at the bottom of the flask. It contained phosphorus in a proportion varying with the amount of enzyme used, the duration of the digestion, and other factors. These results were fully confirmed by Klinkenberg [1882] and Milroy [1896].

The analogy between this precipitate and the insoluble "nuclein" produced from nucleoprotein when acted upon by pepsin had early been recognised, and to avoid confusion the term "paranuclein" had been suggested for the former by Kossel [1891] and "pseudonuclein" by Hammarsten [1893]. Kossel [1886] had definitely proved that the similarity was one of physical features only, for this substance contained no carbohydrate group or purine bases, whereas the true nucleins invariably showed evidence of these bodies on hydrolysis.

Up to the year 1893, when Salkowski commenced his experiments, it was believed that the whole of the caseinogen phosphorus was to be found in the paranuclein, and in Hoppe-Seyler and Thierfelder's *Handbuch* it is so stated. Szontagh [1893], on the other hand, maintained that paranuclein yielded to

prolonged peptic digestion, undergoing re-solution with the separation of some phosphorus as phosphoric acid. This latter fact Salkowski and Hahn [1895] were unable to confirm, although they amply proved that if sufficient pepsin be present practically the whole of the precipitate may go into solution. The re-solution of the phosphorus was also noted by Krehl and Matthes [1895], Alexander [1898] and by Willdenow [1893], although v. Moraczewski [1895] was of the opinion that a small fraction always remains undissolved.

The great variability in the phosphorus content of paranuclein led Robertson [1907] seriously to question its individuality. Values were to be found in the literature ranging from 0·88 to 6·86 % of phosphorus and as a result of his own work Robertson concluded that from the paranuclein first formed a soluble substance containing organically combined phosphorus was split off, leaving a residue of lower phosphorus content (*ca.* 1·8 %).

Salkowski [1899] found that prior to the precipitation of paranuclein the first action of pepsin was so to transform caseinogen that it was no longer precipitable by acetic acid; the separation of the paranuclein then began gradually.

Attempts to isolate individual phosphorus-containing substances from peptic digests of caseinogen have been made by Salkowski [1901], Reh [1907] and Dietrich [1909], but in no case can it be said that a pure product has been obtained.

Action of trypsin. The ability of trypsin to liberate phosphorus during the digestion of phosphoproteins has received much less attention. Sebelien [1895] was the first to conduct experiments of this nature and as a result he stated that in this case no paranuclein was formed, the whole of the caseinogen, except for a negligible residue, going into solution. He did not, however, investigate the form in which the soluble phosphorus was present.

This was done by Biffi [1898] who found that only a fraction (27 %) of the soluble phosphorus could be precipitated by magnesia mixture. The figure was somewhat variable, being influenced by the concentration of enzyme and duration of digestion. Plimmer and Bayliss [1906] confirmed the results of Biffi, and found, on the average, 35 % of the phosphorus present as phosphoric acid after tryptic digestion. These authors also studied the rate at which the phosphorus of caseinogen became soluble (*i.e.* not precipitated by tannic acid) under the influence of trypsin, and found that it was very rapid, the production of phosphoric acid occurring at a considerably slower speed.

Plimmer and Bayliss made no direct attempt to isolate the soluble organic phosphorus-containing substance although they investigated the action of various agents upon it. Thus 1 % sodium hydroxide was found to split off 50 % of its phosphorus as phosphoric acid in 40 hours at 38°. In later experiments Plimmer [1913] found that the whole of the phosphorus could be converted into phosphoric acid under these conditions. Intestinal extracts were also found to have a hydrolytic action upon the organic phosphorus compounds of the digest.

Action of sodium hydroxide. The action of 1 % sodium hydroxide upon caseinogen at 38° for 24 hours was found by Plimmer and Bayliss to result in the liberation of the whole of the phosphorus of the protein as free phosphate, precipitable by magnesium citrate mixture.

So far as we can discover the literature since this date contains no new fact of importance concerning this aspect of the breakdown of caseinogen, and there is in particular no mention of the action of phosphatases on the partially hydrolysed intermediate compounds derived from caseinogen.

Experimental.

The methods employed for the determination of inorganic phosphate in the experiments described in this paper were either the well-known colorimetric method of Briggs, or, where this was inapplicable, precipitation of magnesium ammonium phosphate with magnesium citrate mixture and weighing as pyrophosphate. In certain protein digests, in which, after precipitation with trichloroacetic acid, a cloudy suspension is produced when acid molybdate is added, it is not always possible to carry out the Briggs technique. A satisfactory precipitant for the interfering substances was sought without success. It was found that some of the commoner protein precipitants inhibit colour formation in a marked manner [Rimington, 1924]. Provided, however, that the amount of turbidity produced by the acid molybdate is minute, it is possible to centrifuge the precipitate off without appreciable loss of phosphorus. With a very turbid reaction mixture this procedure will not give accurate results, and it is better, in all cases where turbidity develops with molybdate, to use citrate mixture. In the experiments in which 1 % caustic soda and caseinogen were left in contact at 37° it was found quite impossible to use the Briggs technique, and the rather less accurate magnesium citrate method was employed.

Action of pepsin on caseinogen.

A 1 % solution of Armour's pepsin yielded on filtration a satisfactory enzyme preparation. Hammarsten's "casein" was dissolved in chloroform water (to which 0·8 cc. *N* soda per g. of caseinogen had been added) to form a 0·47 % solution, and portions of this, adjusted to a p_H of 1·2 were incubated in stoppered test tubes at 37° in presence of chloroform. From time to time duplicate tubes were removed and the inorganic phosphorus determined. Our results indicate that no measurable amount of free phosphate is liberated within 9 days, although at the end of this time all the caseinogen has been converted into a form not precipitated by trichloroacetic acid. In this, our findings confirm those of previous workers [Salkowski and Hahn, 1895; Plimmer and Bayliss, 1906].

Bone phosphatase prepared by the method of Kay and Robison [1924] had no hydrolytic effect on the soluble phosphorus compounds in this digest at p_H 8·6. Paranuclein, prepared by the action of 0·25 g. of Armour's pepsin on

1 litre of 2 % caseinogen solution in hydrochloric acid at a p_H of about 1·2, was found to contain 1·4 % of phosphorus. It was dissolved in dilute caustic soda, and the p_H adjusted to 8·6. Bone phosphatase again displayed no hydrolytic activity.

Action of trypsin on caseinogen.

Various aspects of the action of trypsin on caseinogen have been studied, and a phosphorus-containing peptone has been separated from the intermediate products of tryptic digestion.

In order to ascertain to what extent the phosphorus of caseinogen was hydrolysed to inorganic phosphate by trypsin, 200 g. of caseinogen and 200 cc. of pancreatic extract (Allen and Hanbury) in a total volume of 2 litres were allowed to digest at 37° and the acid-soluble phosphorus and free phosphoric acid were determined at intervals in aliquot portions. The following figures show that after over 2 months hydrolysis is not complete:

Days	Soluble P in 10 cc. (mg.)	Free P in 10 cc. (mg.)	% of soluble P as inorganic P
0	0·8	0·8	—
14	6·02	2·47	41·1
73	6·03	3·44	57·1

Total P 6·05 mg. in 100 cc.

The rate at which the acid-soluble phosphorus increases in the earlier stages was determined in another experiment, in which the amino-nitrogen was also followed by the method of Van Slyke. In this experiment 40 g. of caseinogen at p_H 8·4 in a total volume of 2 litres were digested by 100 cc. of pancreatic extract. The results are shown in Fig. 1.

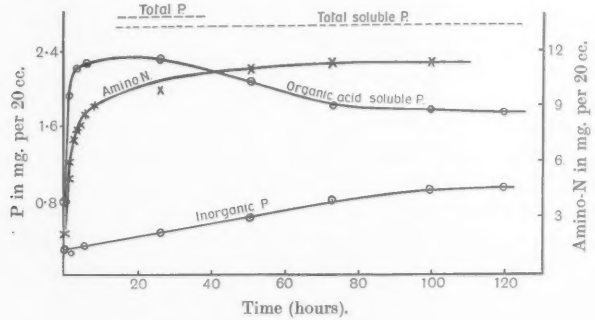


Fig. 1. Action of trypsin upon caseinogen.

During the first 3–5 hours the phosphorus, still in organic combination, is removed from the protein at a rate comparable with that at which amino-nitrogen is produced. After this period, the amino-nitrogen continues to increase whilst the organic phosphorus more slowly approaches a maximum and then begins to diminish. Production of free phosphoric acid takes place

throughout, but at a considerably slower rate, and after several days the organic acid-soluble phosphorus is evidently suffering slow hydrolysis.

With a relatively higher concentration of enzyme it is possible to remove all the phosphorus of caseinogen in an inorganic form. For this purpose pancreatic juice activated by a trace of enterokinase was used. Controls were set up in which the same very low concentration of enterokinase was present as in the pancreatic juice. The substrate was a 0·5 % solution of caseinogen in borate buffer at p_H 8·4, and in presence of chloroform. Fig. 2 shows the results obtained.

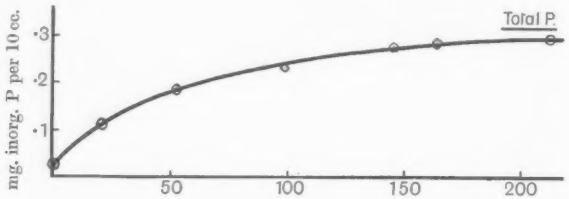


Fig. 2. Action of pancreatic juice on caseinogen at p_H 8·4.

According to Plimmer and Kaya [1909] pancreatic juice itself contains phosphoprotein and nucleoprotein. A phosphorus determination on a sample of pancreatic juice revealed a content of only 0·008 mg. P per cc., which was negligible in comparison with the quantity of phosphorus derivable from the caseinogen.

Hence, in modification of what has been formerly surmised, the organic phosphorus compounds liberated from caseinogen by trypsin are not entirely stable towards this enzyme, and may be almost completely decomposed if the enzyme concentration is sufficiently great and the digestion prolonged.

We could find no account in the literature of any serious attempt to isolate the organic phosphorus compound or compounds produced during tryptic hydrolysis of caseinogen, and it appeared to us not unlikely that some hint as to the mode of combination of the phosphoric acid group in the protein molecule might be obtained from the study of this compound. We have isolated a diffusible substance which contains over 60 % of the organic phosphorus of the parent caseinogen, has strongly acidic properties, forms insoluble salts with the heavy metals, but a soluble barium salt, and has all the reactions and properties of a peptone. It contains 10·5 % N and 3·8 % P[1]. It is hoped to publish, in the near future, the results of an investigation of the properties and composition of this substance, which we call, for convenience, "phosphopeptone."

Action of enzymes on phosphopeptone. The action of *trypsin* upon isolated phosphopeptone is to liberate the whole of the phosphorus as inorganic phosphate in 6 days.

[1] *Note added Aug. 4th.* On further purification the P content rises to 4 %.

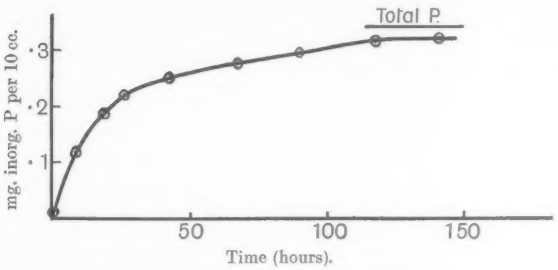
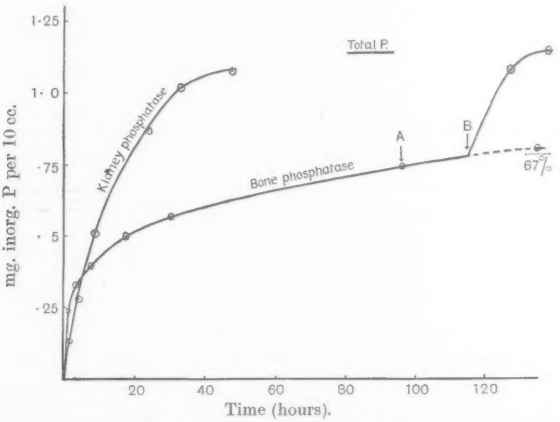


Fig. 3. Action of trypsin on phosphopeptone.

After 9 days at 37° an active *pepsin* preparation had liberated no inorganic phosphoric acid from the phosphopeptone.

Bone phosphatase (rat or rabbit), which is quite unable to liberate phosphoric acid from caseinogen, has a marked action on the phosphopeptone. Two-thirds of the phosphorus of this substance is readily split off, after which any further hydrolysis appears to take place with extreme slowness.



At A a further quantity of bone phosphatase was added.
At B kidney phosphatase (rabbit) was added.

Fig. 4. Action of bone and kidney phosphatase on phosphopeptone.

Kidney phosphatase, obtained from the pig's kidney by maceration with 19 times the weight of chloroform water, displays exactly the same behaviour, but quite a different effect is produced by the phosphatase of the kidney of the rabbit. With this preparation 100 % of the phosphorus of phosphopeptone is liberated as phosphoric acid and the time-curve illustrating the hydrolysis is dissimilar from that afforded by bone phosphatase. In Fig. 4 is shown the course of the latter reaction and, for comparison, the curve obtained when kidney phosphatase (rabbit) is allowed to act upon the same phosphopeptone

solution. At the points indicated fresh enzyme was added and the additional 33 % hydrolysis due to kidney enzyme is seen to take place in a manner closely resembling that observed when this alone is present from the start.

It would appear, therefore, that the phosphatase of bone and rabbit kidney are not identical in nature [see also Kay, 1926] and that phosphopeptone either comprises two different substances, or has two different linkages by which phosphorus is held in organic combination.

Diluted intestinal juice, obtained by washing out the small intestine of a rabbit with 0·9 % sodium chloride and filtering through cotton wool, brings about a fairly rapid conversion of the organic phosphorus both of caseinogen and of phosphopeptone into inorganic phosphate. With caseinogen the process is almost complete in 98 hours, with phosphopeptone complete in 48 hours.

Extract of intestinal mucosa (5 % aqueous) brings about a slow liberation of inorganic phosphorus from caseinogen, but rapid hydrolysis of phosphopeptone, 60 % of the phosphorus of the latter compound appearing as inorganic phosphate in 10 hours. The hydrolysis of the remaining 40 % is a slow process, being almost completed in 5 days. It may therefore be concluded that the organic phosphorus of the phosphopeptone is present in two forms, one fairly easily attacked by phosphatases, the other relatively resistant. All the organic phosphorus of caseinogen is very resistant to phosphatase activity. Thus one of the effects of trypsin upon caseinogen is to render its phosphorus far more readily hydrolysable.

None of the phosphatase preparations—bone, kidney or intestinal mucosa—is capable of removing phosphoric acid from paranuclein (prepared by the action of pepsin upon the same caseinogen) in more than minimal quantities, thus demonstrating another marked difference between the linkages attacked by the two proteolytic enzymes.

Action of alkali on caseinogen.

In investigating the action of alkali upon caseinogen we have adopted the following procedure. A specimen of caseinogen is prepared from separated milk by the usual method, employing acetic acid as precipitant and reprecipitating from a solution of the crude product in dilute sodium hydroxide. Care is taken that the protein shall on no occasion be exposed to strong alkali, never more than the minimum quantity necessary to dissolve the caseinogen (0·8 cc. *N* sodium hydroxide per g. caseinogen) being added to the water in which solution is to be effected.

The product after washing with alcohol is extracted by ether for 6 hours, then dried in a vacuum desiccator. The white powder is then easily soluble in alkali of the above-mentioned strength and contains traces only of ash. A 2 % solution of this caseinogen is made and at the same time a solution of sodium hydroxide; both are warmed to 37° and then equal volumes mixed so that the final solution has a concentration of alkali of 1 %. A sample is taken at once for the zero reading, toluene added and the reaction mixture placed in

a water-bath at 37°. Phosphoric acid is determined in aliquot portions at intervals by magnesium citrate mixture, after removing most of the protein by precipitation with dilute sulphuric acid. The whole of the caseinogen phosphorus becomes liberated as phosphoric acid within 24 hours. By determining the total amino-nitrogen of the solution at intervals, an endeavour was made to find out whether a corresponding increase in free NH_2 groups accompanied the liberation of inorganic phosphorus. A large increase in substances titratable by Sørensen's formaldehyde method did actually occur, but there was a strong smell of ammonia in the reaction flask. In further experiments concurrent determinations of ammonia and amino-groups (Sørensen) were made. Ammonia was determined by drawing a fairly rapid current of air through the reaction mixture at 37° (with precautions to avoid frothing) and absorbing the ammonia in standard acid which was titrated at hourly intervals (Fig. 5). The liberation of ammonia was found to take place in the early stages in a linear manner and is sufficient to account for the increase in the formaldehyde titration figures, within experimental error. At the end of 30 hours, by which time the liberation of phosphorus is complete, ammonia formation is still proceeding with only slightly diminished velocity. It is therefore unlikely that the two processes are directly related.

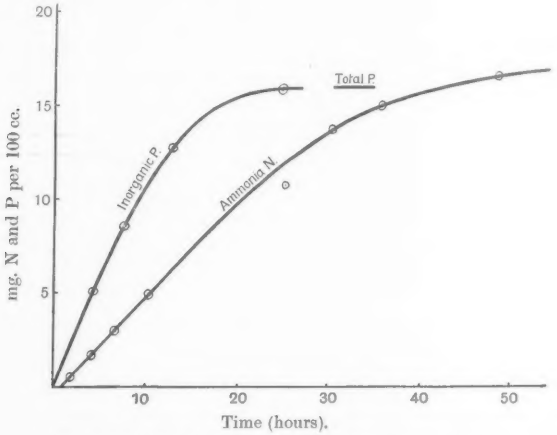


Fig. 5. Action of 1% sodium hydroxide on caseinogen.

During the action of 1 % caustic soda on phosphopeptone, there is also a rapid liberation of phosphoric acid, accompanied by a gradual increase in amino-nitrogen (or ammonia) as determined by Folin's colorimetric method. In the early stages the nitrogen and phosphorus are liberated in the proportion of about 3 P : 1 N, but amino-nitrogen is still being liberated after all the phosphorus has been set free. During the action of bone phosphatase on phosphopeptone there is no increase in amino-nitrogen as measured by

formaldehyde titration. The evidence on the whole does not favour the view that a linkage exists in the caseinogen molecule by which phosphorus and nitrogen are directly associated.

Dephosphorised caseinogen.

Plimmer and Bayliss [1906], and Maynard [1919], remarked upon the opalescence of the solution obtained when 1 % sodium hydroxide acted upon caseinogen, and stated that upon acidification a substance was precipitated which resembled the original caseinogen in its solubility in alkali and insolubility in dilute acetic acid.

This substance may best be prepared by adding acetic acid (33 %) drop by drop to a 36-hour digest of 1 % caseinogen in 1 % soda until maximum precipitation is obtained. The yellowish gummy material separating out is filtered off through muslin, washed, redissolved in sodium hydroxide and reprecipitated; again washed and dried as far as possible on a Buchner funnel (using muslin); it is then transferred to a mortar and ground up with absolute alcohol. At first gummy, it becomes more friable on grinding. The gummy change we were unable to avoid by using alcohol of gradually increasing strength during the dehydration. The final product is a fine white powder, very similar to caseinogen in appearance and properties but slightly hygroscopic. It gives all the protein colour reactions given by caseinogen, but contains traces only of phosphorus and we have called it, for the time being, "dephosphorised caseinogen." The yield from three different specimens of caseinogen is about 40 %. There is a variation in composition according to the previous history of the caseinogen used, but fairly constant figures are obtained from different digests of the same caseinogen. Thus from "Glaxo physiological casein" two different preparations of dephosphorised caseinogen gave the following figures:

	Sample 1	Sample 2
Moisture lost at 107° (% of wet weight)	7·68	5·55
Total nitrogen (Kjeldahl, % of dry weight)	13·17	13·14
Total phosphorus (Neumann, % of dry weight)	0·019	0·019

The caseinogen used for preparation of "sample 2" had been extracted in a Soxhlet for 6 hours with ether.

The low content of nitrogen is a remarkable feature of dephosphorised caseinogen, the accepted figure for the nitrogen of caseinogen itself being 15·65 %.

The explanation of this difference is almost certainly the liberation of ammonia which occurs during the reaction. Schutzenberger [1875, 1, 2] early showed that during the alkaline hydrolysis of caseinogen quite a large evolution of ammonia occurs and Griggs [1921], in a study of the hydrolysis of caseinogen by alkali under pressure at a temperature of 150°, has further found that the amide-nitrogen continues to increase slowly in the later stages probably at the expense of the amino-nitrogen. Sakaguchi [1925], using less drastic conditions, finds that sodium hydroxide not only attacks amide linkages (such

as were shown to be present by Luck [1924]), but also hydrolyses the arginine group in the caseinogen to ornithine. His residual product was found to possess only 0·54 % of its nitrogen in the form of ammonia-nitrogen instead of the 10·3 % of the total nitrogen which is present as ammonia-nitrogen in caseinogen. The composition of dephosphorised caseinogen is being investigated and also that of the albumoses which remain behind in the digest after acidification, but are precipitated by saturation with ammonium sulphate.

Discussion.

The fact that neither bone nor kidney phosphatase has any appreciable action upon the phosphorus of caseinogen, whilst a rapid separation is brought about by these agents of phosphoric acid from phosphopeptone—the substance containing organically bound phosphorus which is produced by the action of trypsin upon caseinogen—suggests that in the latter, linkages are present comparable to those found in hexosemono- and hexosedi-phosphoric acids, α- and β-glycerophosphates, yeast nucleic acid, etc., compounds all of which readily lose their phosphorus as phosphoric acid under the action of phosphatase preparations. The linkages present in caseinogen cannot, however, be of this type. The phosphorus group must be modified in some way, or shielded from the attack of the enzyme.

Both pepsin and trypsin are able to remove the phosphorus of caseinogen, still in organic combination, from the remainder of the protein, but the substances so produced are quite different in their properties, thus affording additional evidence that caseinogen is attacked in completely different ways by the two proteolytic enzymes. The phosphorus-containing substance in peptic digests is not hydrolysed with liberation of phosphoric acid by phosphatase preparations, neither does it yield phosphoric acid under the prolonged action of pepsin. On the contrary, that present in tryptic digests is attacked by both bone and kidney phosphatase, and it is also hydrolysed, more slowly, by trypsin itself, until eventually all the phosphorus appears as phosphoric acid.

The nature of the difference existing between the organic phosphorus as it occurs in caseinogen and as it is present in phosphopeptone after hydrolysis of the protein by trypsin is not yet clear, although the structural modification produced by the enzyme is probably not radical in nature. Thus both compounds display similar behaviour towards alkali (1 % caustic soda at 37° removing the whole of the phosphorus from both caseinogen and phosphopeptone as phosphoric acid within a few hours), towards 1 % acid at 37° and towards pepsin (neither reagent being able to bring about the liberation of phosphoric acid from either caseinogen or phosphopeptone). An additional fact brought to light as a result of the study of the action of phosphatases is that the phosphatases present in rabbit kidney and rat bone respectively have not similar actions upon phosphopeptone, the former removing all the phosphorus of this substance as phosphoric acid, whilst the rat bone preparation

effects only about 66 % hydrolysis. A dissimilarity of the two phosphatases is also indicated by their differing activity towards the phosphoric esters in the blood [Kay, 1926]. It would appear therefore that the phosphopeptone fraction is either a mixture of two or more substances containing organic phosphorus linked in different ways, or that such different linkages exist together in the same molecule. Of necessity these considerations must apply with equal force to caseinogen itself[1] from which the phosphopeptone is derived.

It would appear unlikely that the diffusible phosphoric esters of milk [Kay, 1925] are derived from caseinogen or are directly related to paranuclein or phosphopeptone. The former are hydrolysed to some 60–80 % by bone and kidney phosphatases, but paranuclein is unaffected in presence of these enzymes. Moreover, 2 % sodium hydroxide only liberates traces of inorganic phosphate from the milk esters in 18 hours at 38°, whilst all the organic phosphorus of the phosphopeptone is liberated by 1 % sodium hydroxide in 24 hours.

The frequency of occurrence of phosphoproteins in the material provided by the maternal organism as food for the growing animal has been pointed out earlier. We have not been able to discover in the literature any definite experiments in which very young animals have been maintained on a diet free from organic phosphorus. The relative difficulty with which the organic phosphorus of caseinogen is converted into inorganic phosphate by the alimentary enzymes and the ready diffusibility of some at least of the intermediate phosphopeptones seem to us to support the view that the possibility of uptake of organic phosphorus by the young animal cannot be completely dismissed. The observation of Keller [1900] that in infants there is a rise in the organic phosphorus of the urine after milk feeding is of significance here.

Summary.

Action of pepsin.

On caseinogen. No inorganic P produced in 9 days. Paranuclein obtained, containing a large proportion of the original caseinogen P.

On phosphopeptone. No inorganic P liberated in 71 hours.

Action of trypsin (activated pancreatic juice).

On caseinogen. Complete hydrolysis of the organic to inorganic P when the enzyme is in sufficient concentration; goes through intermediate stage of phosphopeptone; process slow.

On phosphopeptone. Separation of P is more rapid than with caseinogen; half of the organic P is liberated in 20 hours, the remainder is then hydrolysed much more slowly; process almost complete in 6 days.

On paranuclein. Slow hydrolysis.

[1] This is, of course, quite apart from the combination of inorganic calcium phosphate with caseinogen ("acidic caseinogen") described by Lindet [1912, 1916] and by Mellanby [1915].

Action of bone or kidney phosphatase.

On caseinogen. No hydrolysis with bone phosphatase. Very slight hydrolysis with kidney phosphatase.

On phosphopeptone. With each of the two preparations of phosphopeptone used, only 67 % of the organic P was hydrolysed to inorganic P by bone phosphatase (rat). Kidney phosphatase (rabbit) produced 100 % hydrolysis.

On paranuclein. No hydrolysis.

Action of 1 % *sodium hydroxide at* 37°.

On caseinogen. Liberation of 100 % of the organic P as inorganic P in 24 hours. Considerable evolution of ammonia also occurs. Acetic acid precipitates "dephosphorised caseinogen."

On phosphopeptone. 100 % of the organic P liberated as inorganic P in 24 hours.

On paranuclein. 100 % of the organic P liberated as inorganic P in 24 hours.

Action of 1 % *sulphuric acid at* 37°.

No inorganic P liberated from either caseinogen or phosphopeptone after 48 hours.

Our thanks are due to Sir F. G. Hopkins for the interest which he has taken in this work. One of us (C. R.) has been in receipt of a grant from the Department of Scientific and Industrial Research which has enabled him to engage in this investigation.

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CI. KIDNEY PHOSPHATASE.

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During the past few years much attention has been directed towards the acid-soluble phosphorus compounds present in muscle and in the blood, and to the related synthetic and hydrolytic systems. There are present in each of these tissues at least two forms of organic phosphorus compounds which are not precipitated on acidification, and which are in equilibrium, *in vivo*, with the inorganic phosphate present. This equilibrium is controlled by balanced synthetic and hydrolytic systems, both of which, by suitable means, can be shown to exist. The synthetic system, which brings about the esterification of inorganic phosphoric acid is, in each case, more closely associated with the living tissue, though not with the integrity of the cells, than is the hydrolytic, and is much more readily destroyed. Embden and his fellow workers found that in the presence of certain salts such as sodium fluoride and calcium chloride, the synthetic activity of fresh muscle, or muscle juice, outweighs the hydrolytic, whilst Lawaczeck [1924] and Martland, Hansman and Robison [1924] have demonstrated the presence of a synthetic system in recently shed blood. Martland [1925] has shown how delicately balanced is the equilibrium between organic and inorganic phosphorus in blood, slight alterations from the normal hydrogen ion concentration of the blood bringing about either synthesis or hydrolysis. Alkalinity favours the former, and acidity the latter.

In only a few tissues of the body has the phosphorus partition been studied closely. It has been recognised for a long time that phosphorus in organic combination is universally present in the cells of the body. Both "lecithin" and nucleic acid, which do not come into the acid-soluble category, have been detected in most of the organs. Some form of acid-soluble organic phosphorus compound also occurs in most tissues and body fluids which have been examined, namely, muscle, blood, bone, liver, kidney, milk, gastric and intestinal mucosa, spleen, mammary and suprarenal gland. It was shown by Kay and Robison [1924] that the acid-soluble phosphorus in red corpuscles was present in at least two forms, one hydrolysable by bone phosphatase, the other stable to this enzyme. Both forms appeared to be esters of orthophosphoric acid. Goodwin and Robison in subsequent work [1924] showed that they were probably not simple esters. Milk also contains organic acid-soluble phosphorus in at least two forms [Kay, 1925] and hitherto unpublished work

by this author has shown that the same is true for freshly excised liver, kidney, spleen, mammary and suprarenal gland. All these tissues contain phosphatases in greater or less amounts. In fact it may be said that in the tissues of the body and probably in every cell (though not necessarily in the body fluids), wherever there is acid-soluble organic phosphorus, there also is a phosphatase. Any comprehensive picture of the cell's activity must therefore find a place for both enzyme and substrate.

The problem of the physiological significance of the body phosphatases is a fascinating one, and several suggestions have been made with regard to it. Robison [1923] thought that the presence of such an enzyme in growing bone might be associated with the deposition of calcium salts in this tissue, and he and his collaborators have since supported this suggestion with a large amount of experimental evidence. The phosphatase is present, however, not only in the bones and in teeth, but also in considerable quantities in the intestinal mucosa and in the kidney. If it is indeed associated with bone formation, there is undoubtedly some other local factor which determines calcium phosphate deposition, otherwise one would expect calcification to begin, if not in the intestinal wall, then in the kidney. Possibly in the kidney (if, *in vivo*, kidney phosphatase is hydrolytically active) the inorganic phosphate, once liberated, is rapidly excreted and so safely removed from the sphere of the reaction, before it can combine with calcium.

Eichholtz, Robison and Brull [1925] have recently put forward the idea that "normally a considerable part, if not all, of the urinary phosphates is derived from the organic phosphates of the serum (plasma?) by a process of hydrolysis in the cells by the kidney enzyme."

It was decided to study a typical tissue phosphatase more closely, since this seemed to be a point of attack which might yield clues to the main problem of the part played by the organic phosphorus compounds in the functional activity of the tissues. In the present communication, work on kidney phosphatase, mainly of an exploratory nature, is described. At the same time, certain aspects of both the endeavours quoted above to find functions for these very active agents, known to be present in most of the essential tissues of the body, have received attention.

The acid-soluble phosphorus compounds in the normal kidney.

Before dealing with kidney phosphatase itself, a few quantitative observations on the acid-soluble phosphorus of kidney tissue (which contains a substrate upon which, presumably, kidney phosphatase acts in the living animal) are of interest.

The inorganic phosphate of the whole kidney increases rapidly if the fresh tissue is kept for a short time before determinations are made. In order, therefore, to obtain as nearly as possible the distribution of acid-soluble phosphorus in kidney tissue *in vivo*, the kidneys of rabbits which had been killed by bleeding were rapidly dissected out, put on an ice-cold plate, de-

capsulated, opened and freed from fat, connective tissue and traces of blood and urine, weighed and ground up as soon as possible with cooled 3 % trichloroacetic acid. After standing 10 minutes, the mass was made up to a definite volume, filtered, and aliquot portions of the filtrate taken for determinations of free, total acid-soluble, and enzyme-hydrolysable organic phosphorus. Bone phosphatase prepared from rat bone was used as the hydrolytic agent in the majority of the experiments. In three of these, kidney phosphatase was also employed and, on the whole, hydrolysed slightly more of the organic phosphorus than did the bone enzyme. In all cases there was sufficient excess of enzyme to bring about equilibrium in 18 hours. The results for normal rabbit kidney tissue are shown in the first part of Table I. In view of the difficulty of completely removing the last traces of fat, etc. from the pelvis of the kidney in the short time available, the results are surprisingly consistent. Both the organic and acid-soluble inorganic phosphorus of normal, adult kidney tissue are maintained during health within fairly narrow limits

Table I. *Acid-soluble phosphorus in the kidney.*

Normal kidney.

mg. P per 100 g. fresh tissue.

Exp.	Inorg. P	Total P	Hydr. P	Non-hydr. P	Remarks
5	29·5	110·0	60·0	20·5	Three weeks old rabbit
			65·3*	15·2*	
6	22·4	88·7	50·3	16·0	Adult
9	23·5	93·2	53·0	16·7	
			51·8*	17·9*	Adult
10 A	22·2	103·2	42·8	38·2	Adult
11 A	23·5	100·8	48·9	28·4	
			59·0*	18·3*	Adult
20	21·8	89·7	46·6	21·3	Adult
Average	23·8	97·6	50·3	23·4	

Effect of autolysis.

7	43·8	97·6	29·8	24·0	Young adult—kidney removed ¾ hour post-mortem
8	57·0	92·8	11·7	24·1	Adult—kidney removed 2·5 hours post-mortem
10 B	60·4	113·6	19·0	34·2	Adult—other kidney of 10 A above. Removed at once, but kept 3 hours at 35° before analysis
11 B	56·3	106·4	30·3	19·8	Adult—other kidney of 11 A above. Removed at once but kept 3 hours at 35° before analysis
			31·6*	18·5	

Effect of previous injury on rate of autolysis.

51 Normal	58·0	98·8	20·3	20·5	
Injured	62·4	96·4	16·2	17·8	See text
54 Normal	69·0	110·0	27·5	13·5	
Injured	79·8	104·0	18·5	5·3	See text
122 Normal	60·4	107·0	39·5*	7·1	
Injured	65·0	110·0	34·5*	10·5	See text

* Kidney phosphatase used as hydrolytic agent. These figures are not included in the averages.

of concentration, as has been found for the corresponding phosphorus values in the blood.

The organic phosphorus hydrolysable by phosphatase, when the greater experimental errors are taken into consideration, is also maintained at a very constant level in the adult kidney[1].

Effect of autolysis and of injury on the acid-soluble phosphorus of the kidney. As with muscle and with liver [Sevringhaus, 1923], but not with unlaked blood the inorganic phosphoric acid increases rapidly on leaving the fresh kidney to stand for a short time, either within or without the body, after death, and that even at room temperatures. The rate of autolysis of organic phosphorus compounds at 37° is distinctly increased in the kidney by post-mortem injury (second part of Table I).

In Exp. 51 (Table I) two kidneys were taken from the same rabbit as aseptically as possible and weighed. One was injured by striking several times with the sterile scissors. Both were put into an incubator at 37° and allowed to remain in an atmosphere saturated with water vapour for 3 hours, when they were taken out, ground up simultaneously with 3 % trichloroacetic acid, and the acid-soluble phosphorus partition determined.

In Exp. 54 a kidney was divided into two approximately equal halves and each weighed. One half was then cut up with sterile scissors into pieces approximately 2 mm. cube and both halves were put into the incubator, with the same precautions against evaporation, for 3 hours at 37° and analysed as before.

Exp. 122 was a repetition of 54, except that the injured half was damaged by a fairly heavy blow from a pestle.

From these experiments it may be concluded that the rapid production of inorganic phosphorus by the autolysing kidney takes place, as would be expected, principally at the expense of the hydrolysable organic phosphorus, and that there is also a small increase in inorganic phosphorus from non-acid-soluble sources (possibly from lecithin). The effect of injury is to accelerate the rate of inorganic phosphorus production at the expense of the hydrolysable organic phosphorus. It is clear that stoppage of the blood supply to the kidney, or mechanical injury of this organ, throws over the equilibrium (which, in the living animal, is maintained at a very constant level of inorganic phosphorus) to the side of rapid hydrolysis.

Is the equilibrium maintained in the kidney, as in the muscle, by a balanced synthetic and hydrolytic enzyme system? So far, in four experiments, although it has been found that sodium fluoride in effective concentrations of from $N/20$ to $N/10$ markedly hinders the hydrolysis of organic phosphorus com-

[1] In the kidney cells, as in muscle tissue, leucocytes and liver cells, there exists some mechanism by which the inorganic phosphorus is maintained well above that in the circulating blood. The resting-level of inorganic phosphorus in 100 g. rabbit's tissue is as follows: blood 5–6 mg., kidney 23·8 mg., liver 40 mg., muscle 95 mg. The resting-level of organic acid-soluble phosphorus is, however, in the rabbit, less variable from one tissue to another. In mg. per 100 g. it is for red cells 87·4, for kidney 73·8, for liver about 100 and for muscle 99.

pounds in fresh, macerated kidney at room temperatures, no definite evidence of synthesis, such as that described by Embden and his collaborators as occurring in the case of muscle, has been observed. This question must therefore be left open for the present.

Kidney phosphatase.

That there is an active phosphatase in the kidney was shown by Plimmer [1913]. Recent work of Tomita [1922], Forrai [1923, 1924], Robison [1923] and Takahashi [1924] has confirmed and extended these findings, and endeavours have been made by these authors to arrange the various tissues of the body in the order of their phosphatase activity. As Demuth [1925, 1] points out, unless care has been taken to examine the phosphatase activity at the optimum c_H for the enzyme, any series of this kind may be misleading. He finds an optimum c_H for the action of kidney phosphatase on sodium hexosediphosphate in the region of p_H 6. This I have not been able to confirm for the action of kidney phosphatase on either hexosediphosphate or glycerophosphate.

A fairly satisfactory separation of kidney phosphatase from the inactive residue may be carried out as follows. The kidney is ground up very thoroughly with sand and chloroform water and the mass allowed to stand overnight at room temperature. The following morning the mixture is again thoroughly ground up and water added until the total volume is 20 cc. for each g. of kidney tissue. It is then filtered slowly through cotton wool and the opalescent filtrate brought to a p_H of 8·9 with a few drops of 10 % sodium hydroxide. (The volume added in this way is negligible.) If the residual tissue, on the cotton wool, is freed from liquid, as far as possible, by suction, again ground up with water, made up to the same volume as before and again filtered, the filtrate still contains a very small amount of enzyme. In one experiment the second extract had between 6 and 7 % of the activity of the first. The residual kidney had a still smaller but definite phosphatase activity. A single extraction removes at least 90 % of the total phosphatase from the kidney. Using the same method of extraction throughout, results obtained, though quantitatively all some 10 % low, are comparable.

Optimum c_H for kidney phosphatase. Using a preparation of pig's kidney, made as described, it has been found that the optimum c_H, with either sodium glycerophosphate, sodium hexosediphosphate, the organic acid-soluble phosphoric ester of kidney tissue, or the similar ester of blood as substrate, is in the neighbourhood of p_H 9·0, usually between p_H 8·8 and 9·2. Phosphatase from rabbit, rat or human kidney gives the same results. It is, of course, necessary to employ buffer solutions, either borate[1] or glycine buffers (Sørensen) cover the range well. This remarkably alkaline optimum, the most alkaline

[1] Although the buffer is present in excess in all the determinations I have made, there is a theoretical objection to the use of borate buffers where glycerophosphate is the substrate, since the formation of the strong glyceroboric acid, which occurs as soon as glycerol is set free during the enzymic actions, might interfere with the p_H.

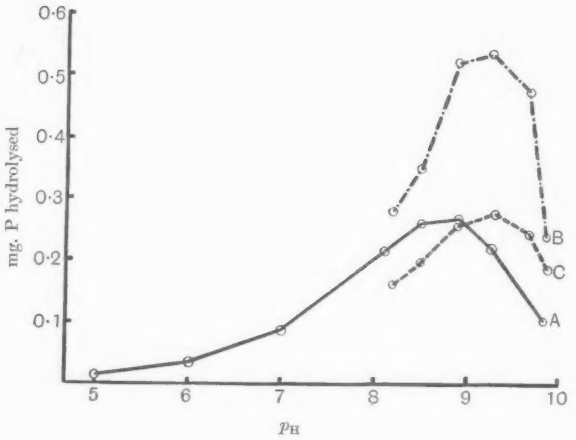

Fig. 1.

Curve A. Action of kidney phosphatase (pig) at differing c_H on sodium hexosediphosphate at 38° (borate buffers).

Curves B and C. Action of kidney phosphatase (pig) at differing c_H on sodium hexosediphosphate (B) and sodium glycerophosphate (C) at 38° (glycine buffers).

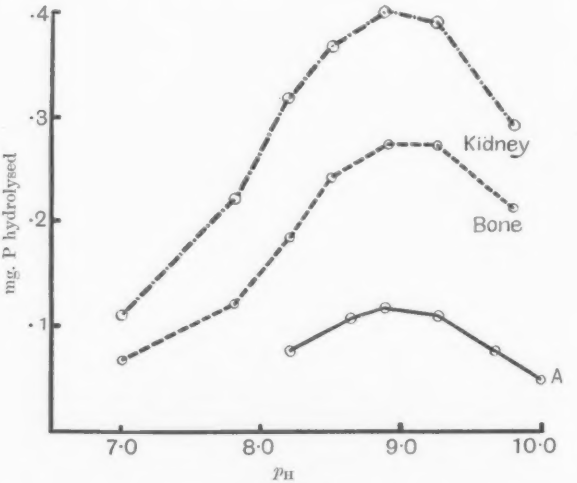

Fig. 2.

Curve A. Action of kidney phosphatase (rabbit) at differing c_H on the acid-soluble organic phosphorus compounds of rabbit's kidney at 38° (glycine buffers).

The other two curves show the action of bone and of kidney phosphatase from the same rat on sodium glycerophosphate at 38° at differing c_H (borate buffers).

yet recorded for any enzyme, agrees with that of bone phosphatase. The significance of this high optimum p_H is by no means clear.

The results of a few experiments which are typical of many others are shown in Figs. 1 and 2.

Using human or rabbit *plasma* phosphatase as the hydrolytic agent, and either glycerophosphate or hexosediphosphate as the substrate, the few experiments made point to the same optimum for this phosphatase also.

Method of estimating phosphatase activity of a tissue. The existence of a fairly broad optimum c_H for both bone and kidney phosphatase renders it possible to estimate with some accuracy the phosphatase content of a preparation of this enzyme. A unit of phosphatase is taken as the amount of enzyme which, at 38° and in Sørensen's glycine-NaOH buffer solution at p_H 8·9, will liberate 1 mg. of phosphorus from excess of sodium β-glycerophosphate solution in 2 hours. By preparing the enzyme solution from tissues in a standard way, one can obtain information as to the relative phosphatase contents of the latter. By this method, the relative activity of bone and kidney of the same animal at various stages of development, the distribution of enzyme in bone and kidney, and the phosphatase content of the kidney in certain cases of disease, have been investigated.

The relative phosphatase content of bone and of kidney. Tissue extracts were carefully prepared, in the standard way just described, from the kidneys and from the bones of a variety of animals, and their phosphatase value determined by the above method. With healthy adult animals, the phosphatase content per g. of whole kidney appears to be fairly constant for each species, but in the young animal the amount of enzyme present varies a good deal with the age. The tissues of the smallest animal investigated—the rat—appear to be most active, but the activity is not inversely proportional to the size of the animal, since the adult pig's kidney is more active than that of the adult rabbit.

Table II. *The phosphatase content of the kidney in adult animals.*

Animal	Phosphatase content (units per g. tissue)	Remarks
Rat	19·2	Average of 4
Rabbit	8·3	„ 2
Pig	12·9	„ 4
Man	About 5	—

Table III shows the change in kidney and bone phosphatase activity with the age of the animal in the case of the rabbit. The same general type of change in the case of kidney phosphatase is true for man; the kidney of the six months foetus is practically inactive, but at term the activity has increased and after birth rises rapidly, probably reaching a maximum before 10 years of age.

Table III. *Development of kidney and bone phosphatase in the rabbit.*

Age of animal	Units per g. tissue: Kidney phosphatase	Bone phosphatase
2–3 days from term	2·6	18·0
New born	4·1	13·7
New born	3·4	15·6
10 days	8·2	15·0
24 days	9·6	11·0
10 weeks	8·2	8·8
Adult (1) 26 weeks	9·4	—
(2) ?	8·2	6·7
Old adult	7·6	1·4

The above results, which are supported by a few figures obtained from the rat, lead to the conclusion that the phosphatase of the kidney, which is low per g. of tissue in the foetal stage, and which increases rapidly just before, and in the few days immediately after, birth, is to be correlated with kidney *function*, and not with the rate of kidney growth. The amount per g. of bone phosphatase on the other hand appears to be a function of the *rate of growth* of this tissue. It is to be expected that as the proportion of inert, inorganic salts in the bone increases, the amount of enzyme present per g. of whole bone will diminish.

In the bone, however, as Robison and Soames [1924] first demonstrated, the enzyme is not equally distributed, and it has been found by the present author that if care is taken to separate the zone lying between the cartilage and shaft in growing bone, where ossification is rapidly proceeding, the activity of this zone is much greater than that of the whole kidney, or the kidney cortex, at any stage of development. The great concentration of phosphatase in this ossifying layer of cartilage has been strikingly shown in the case of a femur from a $6\frac{1}{2}$ months human foetus, confirming the results of Martland and Robison [1924]. In this femur, although the ossifying zone was not completely separated from the cartilage (which was quite inactive) before determining its phosphatase content, it was far more active, weight for weight, than the kidney of the same foetus, or than any human kidney which has been examined (see Table IV).

Table IV. *Relative phosphatase content of bone and kidney.*

Tissue	Phosphatase content (units per g. of tissue)
Foetal kidney	1·0
,, cartilage	nil
,, shaft	6·2
,, ossifying zone (some inactive cartilage present)	12·6
Most active human kidney	5·8

As concerns its phosphatase the ossifying zone in foetal cartilage is far more active than the kidney, and is in this respect probably the most active tissue in the entire foetal body.

The distribution of phosphatase in the kidney. The enzyme was found to be present in much larger amount in the cortex than in the medulla, in all cases examined.

Pig's kidney. Two portions of cortex were taken, one from the peripheral portion, the other from the inner portion adjoining the medulla. A portion of the medulla, mainly consisting of pyramids, was also worked up. The phosphatase content of each portion was as follows in phosphatase units per g. of tissue:

Outer cortex	14·2
Inner cortex	14·2
Medulla	2·1

The enzyme is thus very unequally distributed in the kidney. When hexosediphosphate was used as substrate, the enzyme preparation from the two portions showed the same relative hydrolytic activity as with glycerophosphate.

In another experiment with pig's kidney, the enzyme derived from the cortex and from the medulla (pyramids excluded as far as possible) was allowed to act, under parallel conditions, on solutions of sodium glycerophosphate and sodium hexosediphosphate, containing the same amount of organic phosphorus per cc. (see Table V).

Table V. *Distribution of phosphatase in pig's kidney.*

	mg. phosphorus hydrolysed in 2 hours	
Enzyme	from glycerophosphate	from hexosediphosphate
Cortex	0·234	0·318
Medulla	0·112	0·156
Ratio $\frac{\text{cortex}}{\text{medulla}}$	2·09 : 1	2·04 : 1

This experiment is quoted since it also provides some evidence that the same enzyme is responsible for the hydrolysis of both glycerophosphate and hexosediphosphate, or, if each is hydrolysed by a separate enzyme, then the relative amount of the two enzymes is the same in each portion of the kidney.

Rabbit's kidney. The cortex and medulla were separated as completely as possible with a scalpel, and each ground up with sand and chloroform water as usual. With such a small kidney separation was not complete, but the relative phosphatase content was found to be: cortex : medulla = 2 : 1.

Human kidney. In one experiment with a normal kidney the phosphatase content of the cortex was about four times that of the medulla.

Stability of kidney phosphatase. Preparation of solid phosphatase.

Temperature of inactivation. The enzyme is comparatively thermolabile. After exposure to a temperature of 60° for 5 minutes, there is 90 % inactivation, exposure to 55° for 5 minutes gives 22 % inactivation, slight inactivation after 10 minutes at 50–51°, no noticeable inactivation after 10 minutes at 47·5°. The experiments were made with the enzyme at p_H 7·0.

Stability on keeping. Kidney phosphatase kept at 0° and at 38° for some days gave the following figures in the usual phosphatase units.

Table VI. *Stability of phosphatase on keeping.*

Time kept (hours)	Phosphatase units	
	Kept at 0°	Kept at 38°
0	11·5	11·5
24	11·3	9·3
96	—	7·7
260	11·1	4·2

The enzyme is thus very stable at 0°.

Effect of autolysis of kidney tissue on its phosphatase content. Pig's kidney was finely minced and an equal volume of chloroform water added. Toluene was added to cover the surface of the mixture. Samples were taken for phosphatase determination as follows: at once, activity 15·6 units; after a week at 38°, 5·3 units; after 3 further days at 45°, activity 0·5 unit. Thus autolysis at 38° slowly inactivates kidney phosphatase. In quantitative work with this enzyme, *e.g.* determination of the optimum c_H for its activity, or determination of the phosphatase content of a tissue, experiments of short duration should therefore be made.

Effect of leaving kidney in the body after death. In order to find out whether material from the post-mortem room may be used without serious error as to initial phosphatase content, a few experiments have been carried out with rabbit's kidneys. It was found in preliminary experiments that the two kidneys of a normal rabbit were about equal in phosphatase content. A rabbit was killed, one kidney taken out at once, and its phosphatase content determined. The other kidney remained in the body for at least 24 hours, was then taken out and the determination repeated on this. The results of three experiments, using glycerophosphate as substrate were:

Rabbit (*a*). 12 % diminution in second kidney after 24 hours.
,, (*b*). 9 % diminution after 30 hours.
,, (*c*). 10 % diminution after 30 hours.

In another experiment, a fresh pig's kidney was divided into two parts, the phosphatase activity determined at once in one, and in the other part after keeping for 60 hours at room temperature (slight smell of putrefaction). The diminution in the second part was again 10 %. In the first 30 hours, therefore, post-mortem changes appear to effect a diminution of some 10 % in the phosphatase content of the kidney.

Preparation of a dried phosphatase. If kidney tissue is finely minced, rapidly dried *in vacuo* and ground up in a mortar to a fine powder, the latter is found to possess considerable phosphatase activity. In one experiment with pig's kidney, the activity per g. of *dried* tissue was 19·2 units, whilst the activity of the same kidney tissue as determined by the standard method, was 12·9 units per g. of *wet* weight, or over 40 units per g. dry weight. There

is thus a loss of activity on drying. Once dried, however, the powder retains its activity almost unimpaired for 2 months, and is thus a convenient source of enzyme of definite strength.

Although fractional precipitation of standard kidney enzyme preparations by alcohol or acetone did not yield portions having increased activity, it was observed that after leaving a kidney enzyme preparation at − 4° for a month, on warming to 0° a precipitate settled out which could be filtered off and washed free from salts and soluble proteins and was found to be highly active. The filtrate was almost inactive. The dried solid was found to have an activity of 21·6 units per g., which was retained in the desiccator for 6 weeks at least. The almost inactive filtrate was found to have no adjuvant action on the dried, washed enzyme, which points to the absence of a co-enzyme for kidney phosphatase. A second preparation of solid phosphatase gave the same result. A portion of this second preparation was dried with alcohol and ether. Its activity was found to have fallen from 19·7 units per g. when dried *in vacuo* to 11·4 units per g. when dried with alcohol and ether.

Action of kidney phosphatase on various organic phosphorus compounds.

Hexosephosphate and glycerophosphate. An extract of kidney tissue hydrolyses hexosediphosphate, hexosemonophosphate [Robison, 1923], saccharosephosphate [Neuberg and Behrens, 1926] and glycerophosphate, all phosphoric esters of polyhydroxy-alcohols, and, at least in the case of sodium hexosediphosphate and sodium β-glycerophosphate, the same enzyme appears

Table VII. *Relative rate of hydrolysis of hexosephosphate and glycerophosphate by kidney phosphatase.*

Exp.	Source of phosphatase	Ratio $\frac{\text{Inorg. P liberated from glycerophosphate}}{\text{Inorg. P liberated from hexosediphosphate}}$
5	Pig's kidney—cortex	0·74 : 1
	,, medulla	0·72 : 1
11	Normal human kidney	0·69 : 1
12	Rabbit's kidney—fresh	0·64 : 1
	,, after standing 24 hours in body	0·71 : 1
88	Rabbit's kidney—fresh	0·73 : 1
30	Rat kidney—fresh	0·78 : 1
33	10 days old rabbit—kidney	0·70 : 1
	,, bone	0·74 : 1
	Same bone enzyme after standing 5 days at 0°	0·65 : 1
40	10 weeks old rabbit—kidney	0·75 : 1
	,, bone	0·76 : 1
40 (a)	24 days old rabbit—kidney	0·70 : 1
	,, bone	0·78 : 1
	Mean	0·72 : 1

to be responsible for these hydrolyses. This is shown by the following considerations:

1. The optimum c_H for the action of kidney phosphatase on both hexosediphosphate and glycerophosphate is the same (Fig. 1).

2. Whether the enzyme is derived from the kidney of the rat, the pig, the rabbit, or man, or even from rabbit or rat bone, the relative speeds at which hexosediphosphate and glycerophosphate are hydrolysed, at the optimum p_H of about 8·9 and in presence of excess of the substrate, is ,within experimental error, the same. If hexosediphosphate and glycerophosphate were hydrolysed by different enzymes, one would not expect the ratio of the amount of the two enzymes present to be so constant (Table VII).

3. If a preparation of kidney phosphatase is allowed to act on (*a*) excess of glycerophosphate, (*b*) excess of hexosediphosphate, (*c*) excess of both present together for the same length of time, the amount hydrolysed in (*c*) is not equal to the sum of (*a*) and (*b*), as would be expected if two separate enzymes, a hexosediphosphatase and a glycerophosphatase, were both present in the solution, but is some intermediate figure. The same is true for bone phosphatase. Thus:

	mg. P in 15 cc.	
Hydrolysed in 2 hours from excess of	Kidney phosphatase	Bone phosphatase
Glycerophosphate	0·120	0·169
Hexosediphosphate	0·188	0·246
Both present together	0·127	0·180

α- and β-Glycerophosphate. For the determination of phosphatase activity, pure commercial "sodium glycerophosphate crystals" was employed. This consists of the sodium salt of β-glycerophosphoric acid. If the two pure salts, sodium α- and β-glycerophosphate are compared, it is found that they are not hydrolysed at the same rate by kidney phosphatase, although the optimum c_H remains the same. β-Glycerophosphate, the form incapable of optical activity, is hydrolysed somewhat more readily than the α-form (Table VIII).

Table VIII. *Relative rates of hydrolysis of α- and β-glycerophosphate by kidney phosphatase.*

	mg. P liberated from 10 cc. solution in 2 hours	
p_H	α	β
8·2	0·316	0·320
8·5	0·384	0·425
8·9	0·425	0·494
9·25	0·422	0·478
9·66	0·262	0·353
10·0	0·119	0·218
Total P in 10 cc. (mg.)	2·25	2·23

Caseinogen. Pig's kidney phosphatase was allowed to act on caseinogen solution for 24 hours at 38°. Under the same conditions, but using sodium glycerophosphate or hexosephosphate of the same content of organic phosphorus per cc. of reaction mixture, at least 95 % of this phosphorus would

have been hydrolysed. With caseinogen as substrate in two experiments with two different preparations, only 5 % and 6·2 % of the organic phosphorus of the protein was liberated as free phosphate. The maximum amount of hydrolysis took place at p_H 6·0. Using phosphatase from rabbit's kidney the hydrolysis of caseinogen was slightly greater, being 11 % after 24 hours at p_H 8·9.

Between p_H 6·0 and 9·66 caseinogen phosphorus is only very slowly affected by kidney extract. The small hydrolysis observed is probably due in each case to the presence of small quantities of proteolytic enzymes in the phosphatase preparation (the action of kidney phosphatase on certain of the hydrolysis products of caseinogen is dealt with by Rimington and Kay [1926]).

Nucleic acids and nucleotides. When compared with the rate at which glycerophosphates and hexosephosphates are hydrolysed by kidney phosphatase, the organic phosphorus of yeast nucleic acid, under the same conditions, is hydrolysed slowly, but definitely, and that of thymus nucleic acid very slowly. In conditions in which sodium glycerophosphate would be almost completely hydrolysed in 6 hours by kidney phosphatase, 64 % of yeast nucleic acid containing the same amount of organic phosphorus was hydrolysed in 6 hours, and 83 % in 18 hours at 38°. Only 13 % of thymus nucleic acid under the same conditions was hydrolysed in 18 hours.

The optimum p_H for the action of kidney extract on yeast nucleic acid is on the acid side of 8·5. It would appear that both a "nuclease" and a "nucleotidase" are present in this extract. It is worthy of note that the nucleic acid of plant origin is more easily attacked by animal phosphatase than is that of animal origin.

Table IX. *Hydrolysis of nucleic acids by kidney phosphatase.*

	mg. P liberated per 10 cc. solution	
Time (hours)	Yeast nucleic acid	Thymus nucleic acid
0	0	0
6	0·372	0·025
18	0·480	0·075
Total P	0·580	0·580

Nucleotides. A mixture of nucleotides prepared from yeast nucleic acid by Jones' [1920] method was found to be hydrolysed by kidney phosphatase only slightly more rapidly than yeast nucleic acid of the same concentration in organic P and of the same c_H. As with yeast nucleic acid, equilibrium was reached at about 80 % hydrolysis.

Presence in blood of substrate for kidney phosphatase. Kay and Robison [1924] showed that in mammalian blood filtrates only a portion of the acid-soluble organic phosphorus was hydrolysed by bone phosphatase. The present author has found that pig's kidney phosphatase hydrolyses these phosphoric esters to about the same extent as the bone enzyme, whereas kidney phosphatase from young rabbits brings about almost complete hydrolysis.

Table X. *Hydrolysis of blood phosphoric esters by phosphatases.*

mg. P per 100 cc. blood.

Exp.	Substrate	Free P	Total P	Org. P	Hydrolysis by pig's kidney phosphatase	Hydrolysis by bone phosphatase	Hydrolysis by rabbit's kidney phosphatase
	Rabbit's blood filtrate	4·8	37·5	32·7	13·4	14·0	—
56	Human blood filtrate (man)	3·1	25·4	22·3	6·6	6·5	21·5
53	Human blood filtrate (woman)	2·8	25·7	22·9	—	6·6	20·3
51	Human blood filtrate (man)	3·2	25·5	22·3	6·6	6·6	—
38	Human blood filtrate (diabetic)	3·7	—	—	—	7·7	16·5

Presence in plasma of substrate for kidney phosphatase. Before one can accept the suggestion of Eichholtz, Robison and Brull [1925] as to the physiological function of kidney phosphatase, namely, that it is, by bringing about the continuous hydrolysis of the organic phosphoric esters of the blood, the agent responsible for the presence of the greater part, if not all, of the inorganic phosphates in the urine, it has first to be demonstrated either (*a*) that phosphoric esters capable of hydrolysis by kidney phosphatase are present in the circulating plasma, or (*b*) that the phosphoric esters known to be present in the formed elements of blood are able by some means [cf. the suggestion of the present author, 1925, p. 443] to come into contact with kidney phosphatase. In this connection, it must not be overlooked that both leucocytes and platelets are rich in organic acid-soluble phosphorus (*i.e.* in addition to lecithin and nucleic acid phosphorus). In experiments in conjunction with Dr F. B. Byrom it has been shown that in the white cells, and also in the platelets, the organic acid-soluble phosphorus is partly of a type readily hydrolysable by tissue phosphatases. The leucocytes have perhaps a more direct metabolic relationship to the tissues than the erythrocytes, and it is conceivable that they take a direct part in phosphorus interchange [*vide* Kay, 1926].

Although the amount of acid-soluble phosphorus in plasma is very small, it has been found possible to show that it is partly hydrolysable by kidney phosphatase, and therefore that the liquid bathing the phosphatase-containing cells contains the substrate for their activity. Both rabbit and human blood plasma have been employed, prepared from oxalated or heparinised blood by centrifuging for 20 minutes to remove all cells (including platelets). The plasma is then precipitated with the minimum quantity of trichloroacetic acid, neutralised and the filtrate concentrated to small bulk and freed from inorganic phosphate by magnesia mixture, then neutralised, diluted and treated with kidney phosphatase, with the usual controls. In two quantitative experiments with rabbit's plasma, one heparinised, the other oxalated, it has been found that the hydrolysable phosphorus of the plasma is 0·39 and 0·47 mg. per 100 cc., *i.e.* about 10 % of the inorganic phosphorus. Three similar experi-

ments with human plasma have given results which show that hydrolysable organic phosphorus is present in still smaller, but quite definite quantities.

Action of kidney phosphatase on whole blood. If kidney phosphatase in 0·85 % sodium chloride solution is added in excess to whole blood, there is only very slight hydrolysis, such as might be expected from the small amount of phosphoric ester in the plasma. The method of experiment is as follows. In each of four 50 cc. flasks are placed 3 cc. of whole blood (heparinised and collected under aseptic precautions) + 3 cc. of kidney enzyme in normal saline. To two of these, 20 cc. of water are added, and at once 4 cc. of 25 % trichloroacetic acid, making the volume up to 30 cc. An aliquot portion of filtrate is then taken for the phosphorus determination. The other two flasks (without additional water) are placed in the incubator at 38° for 2–3 hours, then taken out, cooled, and water and trichloroacetic acid added in the same way. Controls with blood and boiled enzyme, with active enzyme alone and with blood and saline alone are carried out under the same conditions. The results are shown in Table XI.

Table XI. *Action of kidney phosphatase on whole blood.*

			mg. P per 100 cc. blood.			
			After period with active enzyme at 38°		After same period with boiled enzyme at 38°	
Exp. no.	At start Free P	Hours	Free P	P hydrolysed	Free P	P synthesised
52	2·6	2½	3·0	0·4	2·1	0·5
26	2·9	3	3·3	0·4	0·7	2·2
28	2·7	3	3·8	1·1	2·3	0·4
20	3·1	3	11·0	7·9	6·8	3·7*

* Hydrolysed.

In Exp. 28 there was very slight laking in the active enzyme flasks, but not in the boiled enzyme flasks, after 3 hours. In Exp. 20 there was marked laking, both in the active and boiled enzyme flasks. In the other two experiments there was no trace of laking. With blood and saline alone there was also a small but definite amount of synthesis.

It is evident from the above experiments that in absence of haemolysis there is:

1. a slight but definite amount of hydrolysis of the phosphoric esters of the whole unlaked blood in presence of kidney phosphatase;

2. in absence of the active enzyme, under the conditions of these experiments, synthesis of organic phosphorus compounds from inorganic phosphate. If there is laking of the corpuscles, the presence of kidney phosphatase markedly increases the rate of hydrolysis.

Effects of certain agents on the rate of hydrolysis of sodium glycerophosphate by kidney phosphatase.

Concentration of substrate. (*a*) With small amounts of enzyme and excess of substrate, the hydrolysis is directly proportional to the amount of the enzyme.

Table XII. *Hydrolysis of excess of glycerophosphate by kidney phosphatase.*

Enzyme added (cc.)	mg. P liberated per 10 cc.	
	Found	Calculated
0·1	0·102	0·096
0·2	0·192	0·192
0·4	0·374	0·384
0·5	0·486	0·480

(*b*) If substrate is not present in excess, the usual type of enzyme curve is obtained:

Table XIII. *Hydrolysis of glycerophosphate by excess of kidney phosphatase.*

Time (hours)	mg. P liberated per 10 cc.
0	0·000
1	0·136
2	0·202
3	0·250
4	0·278
6	0·310
24	0·330
	Total P 0·338

(*c*) With constant small amounts of enzyme there is an optimal concentration of glycerophosphate for hydrolysis at about $M/40$.

Table XIV. *Hydrolysis of glycerophosphate by kidney phosphatase.*

Concentration of glycerophosphate in reaction mixture (M)	Relative rate of hydrolysis in 2 hours
0·2	74
0·13	79
0·10	84
0·05	94
0·033	97
0·025	100
0·016	98·5
0·004	74

Concentration of glycerol. With constant amount of enzyme, increasing amounts of glycerol in the reaction mixture above about 0·8 M diminish the rate of hydrolysis. There is some evidence of a not very marked optimum at about 0·8 M concentration of glycerol.

Concentration of other alcohols. With a constant amount of enzyme present, the addition of increasing amounts of alcohol to the reaction mixture increasingly inhibits the hydrolysis of glycerophosphate. This is true of all the lower alcohols up to butyl and *iso*-butyl. Amyl alcohol is not sufficiently soluble in the reaction fluid to show much inhibition. The percentage inhibition with methyl, ethyl and *n*-propyl alcohol in concentrations from 4 % to 16 % by volume of the reaction mixture is roughly proportional to the concentration of alcohol present. Below 4 % of alcohol by volume the inhibition is very small. Above 16 % analytical difficulties begin to interfere. The inhibitory effect increases as follows: ethyl, methyl, propyl, *iso*-butyl, butyl alcohol.

With the butyl alcohols, insolubility prevents the use of high concentrations.

Action of hormones. Insulin, pituitrin and adrenaline have been investigated and no effect with any of these agents has been observed on the rate of hydrolysis of hexosediphosphate or glycerophosphate by kidney phosphatase. This agrees with the findings of Demuth [1925, 2] and supports the view that hormones which influence carbohydrate equilibrium do not bring about their characteristic effect by directly influencing the activity of enzymes which hydrolyse hexosediphosphate. The optimum c_H of kidney phosphatase is not affected by any of these agents [cf. Langfeldt, 1921].

Dialysis. Preparations of kidney phosphatase have been dialysed in collodion sacs against water. The membranes were impermeable to proteins, but permeable to methylene blue. When dialysed for 18 hours against an equal volume of water, there is no change in the activity of the enzyme solution, and the dialysate remains inactive. On dialysing for a further 18 hours against running water, there is a 5 % loss of activity of the enzyme within the sac.

It may be concluded that kidney phosphatase does not dialyse, nor does it lose its activity on losing the salts and substances of low molecular weight associated with it. The enzyme solution after 18 hours' dialysis against tap water was free from phosphates and contained only as much chloride as tap water. No dialysable co-enzyme is necessary for phosphatase action.

Kidney lecithinase.

There is in kidney extracts, and therefore in kidney tissue itself, a weak lecithinase, which appears to be a different enzyme from kidney phosphatase.

The action of such an enzyme on lecithin is more complex than that of kidney phosphatase on an ester like glycerophosphoric ester, since in order to liberate the free phosphoric acid, both the glycerol residue and choline must be separated from it. This reaction has not been studied in detail. The optimum c_H for kidney lecithinase has been determined, and some idea of the amount present in kidney extracts obtained.

"Lecithin" (Duncan) (0·5 g.) was dissolved in alcohol (20 cc.) and the boiling solution poured slowly into 180 cc. of boiling water. This gives a stable emulsion of lecithin, containing about 0·9 mg. P per 10 cc., less than 1 % of this phosphorus being in inorganic form.

Preliminary experiments showed that the optimal p_H for the lecithinase action of kidney tissue extracts was between 7·0 and 8·0. On repeating the determination (using phthalate and borate buffers) it was found that the optimum was between 7·0 and 7·4, *i.e.* about the normal reaction of the tissue.

The amount of kidney extract which set free 0·3 mg. P from lecithin at the optimum p_H of 7·4 liberated 17·1 mg. from excess of sodium glycerophosphate at the optimum p_H, for the latter hydrolysis, of 8·9. The standard kidney extract contains, therefore, only a relatively weak lecithinase.

Table XV. *Optimum c_H for kidney lecithinase.*

2 cc. kidney phosphatase (prepared in the standard way) to each 10 cc. of lecithin emulsion. mg. P per 10 cc. lecithin emulsion after 60 hours' hydrolysis

p_H	Free P Active enzyme and lecithin	Free P Boiled enzyme and lecithin	Hydrolysed
6·0	0·206	0·159	0·047
7·0	0·291	0·161	0·130
7·4	0·299	0·157	0·142
7·8	0·278	0·161	0·117
8·2	0·272	0·163	0·109
9·25	0·242	0·163	0·079

Total phosphorus in 10 cc. of lecithin emulsion 0·900 mg.

The source of urinary phosphates.

It is possible, in view of certain of the results of the paper, to examine in a roughly quantitative way the view of Eichholtz, Robison and Brull [1925] that the organic phosphoric esters of the plasma may provide a large part of the phosphate of the urine.

The daily excretion of urinary phosphorus by the normal adult is about 1·2 g. From the activity of human kidney phosphatase (approximately 5 phosphatase units per g. kidney at p_H 8·9) and assuming the active tissue to be 300 g. it would be possible to liberate 5×300 mg. P from glycerophosphate in 2 hours or $5 \times 300 \times 12$ in 24 hours = 18 g. and considerably more from hexosephosphate. The rate of hydrolysis of the blood esters is, under similar conditions, not far short of that of glycerophosphate. But we have no reason to suppose that the phosphatase of the kidney is working, *in vivo*, at other than the p_H of the blood, *i.e.* 7·4. At this p_H its phosphatase activity is from one-third to one-half of its activity at the optimum p_H. However, taking the lower value of one-third, which would bring the excretion down to 6 g. of phosphorus from glycerophosphate and somewhat less from the blood esters, there is quantitatively a large excess of phosphatase.

From the experiments quoted in this paper, the limiting factor would appear to be not the amount of phosphatase, but the quantity of substrate in the *plasma*. (We cannot assume that the phosphoric esters in the corpuscles diffuse into the plasma *in vivo* until it has been shown to be possible *in vitro*, and so far every endeavour to get the phosphoric ester out of the corpuscles, short of laking them, has been unsuccessful.) It has been shown that the greater portion, at least of the organic acid-soluble phosphorus of the plasma, is hydrolysed by kidney phosphatase *in vitro*. If we assume this amount to be 0·3 mg. per 100 cc. of blood (probably an over-estimate), it seems at first that the normal amount of phosphorus excreted in the urine in 24 hours—1200 mg.—is very large compared with this concentration. If all the plasma organic phosphorus present in the entering blood were hydrolysed during each passage through the kidney, and if the plasma level were maintained by some synthetic mechanism in another part of the organism, 16 litres of blood per hour would have to be completely relieved of its plasma organic phosphorus

by the kidney. It has been computed that some 900 litres of blood pass through the human kidneys in 24 hours, an average of just under 40 litres per hour, so that it is quantitatively possible for the whole of the inorganic phosphate of the urine to come from the organic phosphoric ester of the plasma.

The close correlation between the level of inorganic P in blood and in urine [cf. Wigglesworth and Woodrow, 1924; Addis, Meyers and Bayer, 1925; Adolph, 1925] could perhaps be explained, on the enzyme theory of phosphate excretion, by the additional hypothesis that increase of inorganic phosphate in the plasma leads, possibly by mass action, to increased synthesis of phosphoric esters and this to the greatly increased excretion of inorganic phosphate. Up to the present, there is no direct evidence available on this point. In a few experiments reported by Wigglesworth and Woodrow no increase, but rather a decrease in the organic acid-soluble phosphorus of *whole blood* was noted, though a simultaneous rise in the organic phosphorus of the plasma is not thereby excluded. The extreme rapidity of the excretion of inorganic phosphate, described by Addis and his collaborators [1925] as following the injection of massive doses of neutral phosphate solutions, is difficult to explain even with the help of the additional hypothesis. It appears on the whole that, before a theory postulating the dependence of inorganic phosphate excretion by the kidney on the level of phosphoric esters in the plasma can be accepted, much more evidence is required.

The occurrence in a particular tissue of an enzyme capable of decomposing a substrate present in the same locality in the body, is not, in itself, sufficient evidence that the two are directly physiologically related. But the presence and peculiar properties of this very active phosphatase in such an important and efficient organ as the kidney, if we reject the hypothesis of Eichholtz, Robison and Brull, demands an explanation. It may be urged that phosphatase activity, not only in the kidney but also in other tissues, is merely the fortuitous result of a particular type of molecular configuration of fairly frequent occurrence in the cell. There is also the view that phosphatase, *in vivo*, acts as a synthetic enzyme, and is connected with the inner life of the cell, in the maintenance of the essential nuclear phosphorus compounds, and is not involved directly in the functional activity of the organ in which it occurs. But as regards the phosphatase in intestinal juice and the mucosa of the gut wall, this is clearly not the case; with bone phosphatase also the correlation between the rate of deposition of calcium in the growing bone and the phosphatase content of the active zone bears strongly against this possibility; further, the correspondence between the functional activity of, and the amount of phosphatase present in the kidney at various stages in the development of the animal, is against its being true for this organ.

Summary.

1. The inorganic phosphate, and to a less degree the acid-soluble organic phosphorus in the kidney remain constant during the adult life of the animal.

2. The rate of production of inorganic phosphate during the autolysis of the whole kidney is increased by mechanical injury to the organ.

3. Kidney extracts contain a very active phosphatase which is the main agent concerned in post-mortem phosphatolysis. It has an optimum p_H of 8·8–9·2, the most alkaline optimum yet recorded for any enzyme.

4. A simple method has been worked out for determining the phosphatase content of a tissue extract, and has been applied to the determination of the phosphatase content of bone and kidney tissue at various stages in the life of an animal.

5. It is concluded that bone phosphatase is correlated with bone *growth*, and kidney phosphatase with the functional activity and not with the growth of this organ.

6. Phosphatase is not uniformly distributed in the kidney, being present in greatest quantity in the cortex.

7. A short autolysis reduces the phosphatase content of the kidney very little. At 0° in presence of antiseptic kidney phosphatase remains for weeks without serious loss of activity.

8. An insoluble phosphatase preparation has been obtained, which, when dry, is stable at room temperature.

9. Hexosediphosphate and glycerophosphate are hydrolysed by the same enzyme. Sodium β-glycerophosphate is hydrolysed more readily than the α-salt. Caseinogen is hardly attacked by kidney phosphatase. Yeast nucleic acid and nucleotides are hydrolysed readily, but thymus nucleic acid only slowly, by this enzyme.

10. Preliminary experiments have been carried out on the effect of certain agents on the rate of hydrolysis by kidney phosphatase.

11. Kidney extracts contain a feeble lecithinase which has an optimum p_H of 7·0–7·4.

12. Both red cells and blood plasma contain a substrate for kidney phosphatase.

13. The kidney phosphatases from different animals vary in their ability to hydrolyse the organic phosphoric esters of the blood.

14. Kidney phosphatase acting on whole blood will hydrolyse the organic phosphorus in the plasma, but is not able to hydrolyse the organic esters within the red cells themselves.

15. The possible physiological significance of kidney phosphatase is discussed.

I am indebted to Dr J. H. Quastel for pure specimens of sodium α- and β-glycerophosphate, and to Mr P. G. Marshall for assistance in some of the later experiments.

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CII. OXIDATIVE MECHANISMS OF TUMOUR TISSUE.

I. THE ANAEROBIC HABIT OF TUMOUR TISSUE.

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Report to the Medical Research Council.

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Warburg has shown in a recent series of papers [Minami, 1923; Warburg, Posener and Negelein, 1924] that the metabolism of cancer tissue may be regarded as resembling in some respects that of an anaerobic rather than an aerobic organism. It is capable of converting glucose into lactic acid with great rapidity, but the oxidative removal of the lactic acid is not so rapid. Thus, if slices of tumour tissue are placed in a Ringer's solution containing glucose, large amounts of lactic acid accumulate even when an abundant supply of oxygen is ensured. The oxidative activity of the tissue does not keep pace with the fermentative, and Warburg found that cancer tissue differed from normal tissues in this respect.

In his first paper on glutathione Hopkins [1921] stated that "actively growing cancer cells seem to show a nitro-prusside reaction of surprisingly low intensity." Sir F. G. Hopkins informs me that this statement was mainly based upon experiments with the Jensen rat sarcoma. Later observations on human material did not seem to confirm it, but this was at a time when no distinction was made between the reaction due to the "fixed SH group" of the tissue, and that due to soluble glutathione.

Callow and Robinson [1925] carried out a series of glutathione determinations on aerobic and anaerobic bacteria, and found the anaerobic to be notably deficient in their content of glutathione.

In view of this work and of the work of Warburg it seemed to be of interest to carry out determinations of the glutathione content of cancerous tissues.

The glutathione was extracted and titrated by the method of Tunnicliffe [1925]. The tissue was repeatedly ground and extracted with 10 or 20 % trichloroacetic acid and filtered, and the filtrate then titrated with $N/100$ iodine, using the nitroprusside reaction as an external indicator. Necrotic tissue was most carefully avoided.

Some typical results are given in Table I. The glutathione content of cancerous tissue is very low indeed for a cellular tissue. For the liver (rabbit and rat) Tunnicliffe obtained a mean value of about 200 mg. per 100 g. wet tissue. Hopkins (unpublished experiments) for the rabbit's liver has obtained values ranging from 250 mg. after 24 hours' starvation to 340 mg. in a well-fed animal. For the kidney, Tunnicliffe's mean figure was 170 mg., while

Hopkins' data show a range from 130–145 mg. Tunnicliffe's figure for muscle is about 35 mg., the amount in muscle being small for a normal tissue.

It will be seen from the table that, in five cases out of seven examined, cancer tissue contained too little reduced glutathione to make estimation possible by the method used. It was in all these cases certainly less than 3·0 mg. per 100 g. tissue. The highest figure for cancer tissue, in the case of a rat sarcoma, was about 16 mg. The dry weight of tumour tissue is not much more than half that of muscle, however, and this, the highest figure and apparently exceptional, if calculated per 100 g. dry weight instead of wet weight, might give a value about equal to that of muscle. The tissues of young embryos, which have also a very high water content, and which, since they are growing tissues, give an interesting comparison, have been found to contain a large amount of glutathione [Murray, 1926]. The contrast with the quick-growing rat carcinoma and the quick-growing human mammary carcinoma is striking.

Table I.

Description of tissue	Glutathione content in mg. per 100 g. wet weight of tissue
Jensen rat sarcoma 2·4 g.	Small trace
„ „ 6·1 „	16·5
„ „ 5·0 „	Small trace
Rat carcinoma 9 2·2 „	„
„ „ 1·4 „	„
Quick growing and cellular human mammary carcinoma	7·5
Scirrhous human mammary carcinoma	Small trace
Rat brain 1·5 g.	50
Rabbit brain—grey matter	75 } 63
„ white matter	52 }
Metastatic nodules in axillary lymph glands from human mammary carcinoma	75

The results given by human tumours correspond well with those given by rat tumours.

The only exception I have so far found to the low values given by tumour tissues is in the case of metastatic growths in lymph glands. In two cases of human mammary carcinoma I have obtained normally high values in infected axillary glands. It has not been possible as yet to determine whether this puzzling answer is due to an increased power of the tissue to reduce its glutathione (the iodine titration estimates, of course, only the reduced form) or whether the metastatic nodules actually pick up or form a larger amount of the substance than the parent tumours.

Similarly it is not yet certain whether the low figures obtained for tumour tissue are due to a low content of glutathione or to a decreased power to reduce the oxidised form. Analyses of the total sulphur in the tissue extracts will be carried out to settle this point.

Warburg [Warburg *et al.*, 1924] showed that the grey matter of the brain and the retina were the only normal adult tissues which could convert glucose into lactic acid as actively as cancer tissue. On the other hand, the grey matter of the brain is also capable of oxidising the lactic acid rapidly. It

seemed, therefore, of particular interest to compare the oxidative mechanisms of nervous tissue and cancerous tissue.

The amount of glutathione in the grey matter of rabbit's brain, even when this is only very roughly divided from the white, seems to be about 75 mg. per 100 g. wet weight of tissue. Values obtained are given in Table I.

Experiments have been carried out on three different tumours to determine to what extent cancer tissue is capable of converting added oxidised glutathione into the reduced form under strictly anaerobic conditions. All normal tissues examined by Hopkins have shown this capacity to a high degree.

About 3–5 g. of tissue were placed in a vacuum tube with 20 cc. of Ringer's solution containing 50–100 mg. of oxidised glutathione. The tube was very carefully exhausted, washed out with nitrogen and re-exhausted. A control tube containing the same amount of tissue and of fluid, and at the same p_H (7·4), but containing no glutathione, was treated exactly similarly. The tubes were then incubated for 3 or 4 hours at 37°.

Under these circumstances a considerable reduction of the oxidised glutathione would normally be expected. The tumour tissue proved to be extraordinarily inactive in this respect. In one case definite reducing power was displayed, but very small compared with normal tissues. The results are shown in Table II with data from normal tissues for comparison.

Table II.

Tissue	mg. of GSH per 100 g. tissue after incubation anaerobically: Without added G_2S_2	With added G_2S_2
Rabbit's liver	297	780
,, kidney	138	1020
Rat sarcoma (*a*)	±1·5	±4·5
,, (*b*)	Trace	±1·5
,, (*c*)	Trace	91

The fact that the tumour tissue can reduce a further amount of oxidised glutathione suggests that, under the conditions of the original estimation, the glutathione already present is all reduced and therefore all estimated. On the other hand, the tissue is quite remarkable in that it only reduces such a very small amount of the added glutathione. In this respect tumour tissue seems to differ from anaerobic bacteria, as these can reduce added G_2S_2. Quantitative comparison of the two is, however, very difficult. A further investigation on these lines should yield very interesting results.

In addition to glutathione, the tissues of rat sarcoma and carcinoma have been examined for the respiratory pigment *cytochrome*. Dr Keilin, who first described this pigment [1924], has very kindly examined the tissues. He has so far always found that aerobic tissues and organisms, and particularly those which might be expected to show great metabolic activity, contain considerable amounts of this pigment, whereas it is lacking in anaerobic organisms.

In order to remove as much haemoglobin as possible from the tumour tissue, so that the cytochrome spectrum might not be obscured, the rat bearing

the tumour was perfused for about 1 hour with Ringer's solution. The animal was killed by chloroform anaesthesia, the heart exposed, and a cannula tied into the left ventricle. Warmed Ringer's solution was allowed to flow through this and eventually to escape through a cut in the right ventricle, after passing through the systemic circulation.

After perfusion, small portions of the tumour and of the grey matter of the brain were removed for examination. The pieces of tumour tissue were taken from the most quickly growing portion of the tumour and necrotic tissue was carefully avoided.

The results of Dr Keilin's spectroscopic examination of these tissues were of very great interest.

The grey matter of the brain was found to contain a high concentration of cytochrome. The tumour tissue, on the other hand, contained very little of the pigment, and the position of the absorption bands resembled that shown by brewer's yeast.

The tissue of the Jensen rat sarcoma was examined twice, that of rat carcinoma 9 once, and the results were exactly similar on all three occasions. The grey matter of the rat brain was twice examined.

These results undoubtedly support very strongly the idea that the cancerous cell may live in some respects like an anaerobic organism. They also emphasise the difference, which was first pointed out by Warburg, between the activities of the cells of the grey matter of the brain and those of cancerous cells.

SUMMARY.

1. The tissues of rat sarcoma, rat carcinoma and human mammary carcinoma have all been found to contain abnormally small amounts of reduced glutathione.

2. Rat sarcoma and rat carcinoma tissue, unlike normal cellular tissue, appear also to show only very slight activity in reducing added oxidised glutathione.

3. Spectroscopic examinations of rat sarcoma and rat carcinoma tissue indicate deficiency in the respiratory pigment, cytochrome.

4. It is thought that these observations support the contention of Warburg, that the cancer cell resembles in some respects an anaerobic rather than an aerobic organism.

I have much pleasure in thanking Professor Gask, Dr Piney, Mr Perrin, Professor Ellis and Dr Byrom for their kindness in providing me with the human cancer material.

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CIII. THE SURFACE TENSION OF GELATIN SOLUTIONS. (PART II.)

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(*Received July 2nd, 1926.*)

In a previous communication [1925] it was shown that the surface tension of ash-free gelatin solutions varied with the p_H of the solution, giving a maximum at the isoelectric point and another maximum about p_H 3·0. Further work was undertaken in an endeavour to find the cause of the fall of surface tension in the more acid solutions, which was not expected from consideration of the other physical properties of gelatin, or from Donnan's membrane equilibrium theory.

de Caro [1925] has confirmed the existence of this second maximum of surface tension at p_H 3·0 in an investigation of the effect of p_H on the drop numbers of 2 % gelatin at 30° as determined with the Traube stalagmometer, but contrary to our results he found the surface tension a minimum at the isoelectric point, and therefrom draws the conclusion that the surface tension is most lowered by the undissociated protein molecule. That this cannot be the case we have previously shown both theoretically and practically and many subsequent determinations have confirmed that the surface tension of isoelectric gelatin solutions rises from an initial low value very nearly to that of water as flocculation proceeds, so that it seems probable that de Caro did not allow sufficient time for flocculation to occur before determining the drop number. This effect of time is less marked at other values of p_H, but all our determinations were made after the solutions had stood at 25° for 24 hours, or when completely flocculated in the case of isoelectric gelatin.

The values for the p_H of our gelatin solutions and also those of de Caro were determined colorimetrically. On checking these values electrically with the quinhydrone and hydrogen electrodes we find the colorimetric method is affected by a larger protein error than was anticipated, and there is a considerable discrepancy, varying with the p_H, between the methods. Consequently it has been necessary to correct our previous surface tension-p_H results to some extent as regards p_H.

The existence of a "protein error" in determining the p_H of protein solutions by means of indicators has been pointed out by Sørensen [1909, 1912], Kolthoff [1921], Clark [1922] and others. Although we find the magnitude

of the difference between the electrical and colorimetric values of the p_H to vary with the state of dissociation of the protein in apparently a regular manner, in view of the complexity of the problem due to other possible sources of error, such as the use of two-colour indicators in the standards, we consider it best merely to refer our results to the experimental values determined electrically, as advised by Clark [1922], for the particular protein and indicators investigated.

As a result of the electrical determination of p_H, it will be seen that the second maximum of surface tension is at a point more acid than originally found, namely about p_H 2·2 instead of about p_H 3·0. This seemed to encourage the idea that the further fall of surface tension in solutions of higher acidity might be due to some effect of the strong acid on the protein, such as hydrolysis or denaturation. An attempt was therefore made to detect hydrolysis by means of formaldehyde titrations for amino-acid, but definite evidence that it was due to hydrolysis of the protein with formation of amino-acids was not obtained.

Experimental.

The gelatin, Nelson's Gold Label powdered, was rendered ash-free as previously described [1925] and in all cases contained less than 0·1 % ash in the dry gelatin.

The surface tension was determined by Sugden's maximum bubble pressure method [1922, 1924]. The colorimetric determinations of p_H were carried out as previously described and the standard buffer solutions checked electrically.

The electrical determinations of p_H were made with the quinhydrone electrode at 20° below p_H 7·5 and above that with the hydrogen electrode. We are indebted to Professor A. R. Ling for the use of the hydrogen electrode.

The discrepancy between the electrometric and colorimetric determinations of the p_H *of gelatin solutions.*

Solutions of 1 % and 0·4 % gelatin with varying amounts of $N/10$ HCl or $N/10$ NaOH were prepared from a 2 % stock solution of isoelectric gelatin and the p_H determined by both methods after allowing to stand at 25° for 24 hours to ensure equilibrium being attained.

Electrometric determinations were repeatable within 0·02 p_H but colorimetric determinations of the apparent p_H were only readable to 0·05 and varied with the observer owing to the difficulty of colour matching, hence the given values are the mean of several independent observations. Owing to the absence of electrolytes it was difficult to obtain a good reading electrometrically for the isoelectric point. The results for 1·0 % and 0·4 % gelatin are given in Table I. Fig. 1 shows the discrepancy, or "protein error," plotted against the p_H as determined electrometrically; the error is very nearly the same in the case of the 0·4 % as in the 1·0 % solution when bromophenol blue is the indicator, but differs somewhat with the concentration for thymol blue.

Table I. *The p_H of gelatin solutions determined colorimetrically and electrometrically.*

A. 1 % *gelatin solution.*

cc. *N*/10 HCl in 100 cc.	Indicator	Colorimetric p_H	Mean	Electrometric p_H	Discrepancy (colorimetric – electrometric)
0	Bromophenol blue	4·7, 4·75, 4·8	4·75	4·86	−0·11
1·7	,,	4·4, 4·4, 4·45	4·42	4·33	+0·09
3·3	,,	4·2, 4·3	4·25	4·03	+0·22
5·0	,,	4·2	4·2	3·65	+0·55
6·7	,,	4·00, 3·95, 4·05, 3·95, 4·05	4·0	3·27	+0·73
10·0	,,	3·6, 3·7, 3·65, 3·55	3·6	2·74, 2·74	+0·86
13·3	,,	3·3, 3·25, 3·2, 3·25	3·25	2·36, 2·38	+0·88
16·7	,,	3·0, 2·9, 2·8, 3·0, 3·0	2·95	2·11, 2·12	+0·83
,,	Thymol blue	2·75	2·75	,,	+0·63
23·3	,,	2·3, 2·3	2·3	1·87	+0·43
33·3	,,	2·0, 1·9	1·95	1·64	+0·31
50·0	,,	1·75, 1·7, 1·7	1·71	1·43	+0·28
cc. *N*/10 NaOH in 100 cc.					
0·8	Bromocresol purple	5·7, 5·6	5·65	5·49	+0·16
1·7	,, / Bromothymol blue	6·3, 6·1	6·2	6·15	+0·05

B. 0·4 % *gelatin solution.*

cc. *N*/10 HCl in 100 cc.					
0	Bromophenol blue	4·8, 4·7	4·75	4·86	−0·11
0·8	,,	4·45, 4·40	4·425	4·21	+0·215
1·7	,,	4·25, 4·2	4·225	3·82	+0·405
3·3	,,	4·0, 3·75	3·875	3·22	+0·655

Table I (*continued*).

cc. $N/10$ HCl in 100 cc.	Indicator	Colorimetric p_H	Mean	Electrometric p_H	Discrepancy (colorimetric – electrometric)
6·7	Bromophenol blue	{3·5 / 3·5}	3·5	2·76	+0·74
8·3	,,	{3·3 / 3·25}	3·275	2·38	+0·895
10·0	,,	{3·0 / 2·9}	2·95	2·20	+0·75
,,	Thymol blue	2·8	2·8	,,	+0·60
13·3	,,	{2·5 / 2·3}	2·4	2·06	+0·34
16·7	,,	{2·2 / 2·0}	2·1	1·93	+0·17
20·0	,,	{2·05 / 1·9}	1·975	1·81	+0·165
23·3	,,	{1·9 / 1·8}	1·85	1·76	+0·09
33·3	,,	1·6	1·6	1·57	+0·03
cc. $N/10$ NaOH in 100 cc.					
0·3	{Bromothymol blue / Bromocresol purple}	{5·9 / 5·7}	5·8	5·38	+0·42
0·6	,,	{6·8 / 6·9}	6·85	6·70	+0·15

In the colorimetric determinations, the matching of the gelatin solutions with the standards was rendered difficult by the difference in quality of the light transmitted through the gelatin solution and indicator from that transmitted through the standard and indicator, although the latter was compensated with another tube of the gelatin solution without indicator placed in front of it. The use of compensating tubes helped to overcome in part the difficulty with a dichroic indicator such as bromophenol blue due to the different absorption of the two colours by the solution, which thus alters the quality of the transmitted light according to the thickness of solution traversed. In the case of a turbid solution this is particularly noticeable as light which has entered from the side of the tube is reflected from the particles, thus all the transmitted light has not traversed the same thickness of solution. Except in the case of isoelectric gelatin, the solutions of both 0·4 % and 1·0 % gelatin were apparently optically clear, so any such reflection should be small and would be greatest near the isoelectric point. Such changes of shade certainly seemed most noticeable with bromophenol blue near the isoelectric point, but the colour change of the indicator with p_H is very marked about there, which considerably aided the matching. Reference to Fig. 1 will show that the discrepancy is least about the isoelectric point.

The use of a colour screen to exclude one of the principal colours, as advocated by Clark [1922], did not seem to improve the accuracy of the matching as it considerably lessened the colour change with p_H of the indicator. As the gelatin solutions are free of neutral salt, there may be a difference in apparent p_H between them and buffer solutions containing the

same indicator, the latter being standardised against the electrical determination for a definite concentration of neutral salt usually $M/10$ or $M/15$. Lepper and Martin [1926] have, however, shown that the magnitude of this difference does not exceed $\pm$ 0·2 between $M/1$ and $M/1000$ for phenol red indicator, but Kolthoff [1921] finds that bromophenol blue is particularly influenced by neutral salt action. The error obviously depends on the indicator used, as shown by Sørensen [1909], *e.g.* we found that bromocresol purple and bromothymol blue are little affected, but consideration of Fig. 1 shows that it depends to

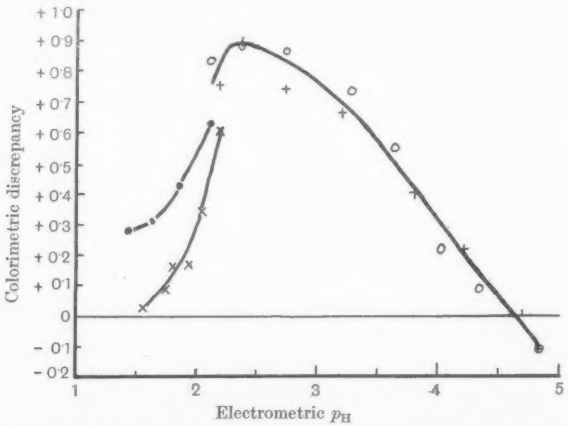

Fig. 1. Variation of the "protein error" in the colorimetric determination of p_H with the p_H as determined electrometrically.

○ Bromophenol blue and 1·0 % gelatin. ● Thymol blue and 1·0 % gelatin.
\+ „ „ 0·4 % „ × „ „ 0·4 % „

a great extent on the state of dissociation of the protein for bromophenol blue and thymol blue. The error is a minimum about the isoelectric point, and a maximum about p_H 2·4 (electrometric). Actually p_H 2·4 is outside the range of bromophenol blue (2·8–4·6), although the solution appears to have the p_H 3·25 and is alkaline to thymol blue (1·2–2·8); hence the error due to this cause is probably increased since indicators work most accurately near their point of 50 % dissociation, which is at p_H 4·1 for bromophenol blue. Evidently the dissociation of the protein affects the indicator by combination or by adsorption to a greater extent when dissociated than when undissociated, thereby upsetting the normal ionic equilibrium of the indicator and hence changing its colour.

The effect of p_H *on the surface tension of gelatin solutions.*

In Fig. 2 is given the effect of p_H on the surface tension of gelatin solutions, the p_H being determined electrometrically. It will be seen that the maximum at the isoelectric point remains unaltered whilst the minimum previously

found at p_H 3·8–4·0 is now at p_H 3·5, and the second point of maximum surface tension is considerably more acid than formerly, namely, p_H 2·0–2·2 instead of p_H 2·8–3·0. The curve of 1 % gelatin in presence of H_2SO_4 does not agree so closely with that of 1 % gelatin in presence of HCl as formerly observed, but the point of minimum surface tension is at exactly the same p_H for each acid. Previously recorded experiments [1925] on the effect of time and of neutral salts on the surface tension of 1 % gelatin in presence of HCl at p_H 3·3 as found colorimetrically were thus actually carried out at an original p_H of 2·37, excluding the effect of neutral salts on the p_H. This alteration, due to the error in estimating p_H, does not affect our previous conclusions, except that the point of minimum surface tension at p_H 3·5–3·6 does not agree so well with the point of maximum potential difference found by Loeb [1922] at p_H 3·8–4·0, though it agrees well with the maxima of osmotic pressure and viscosity.

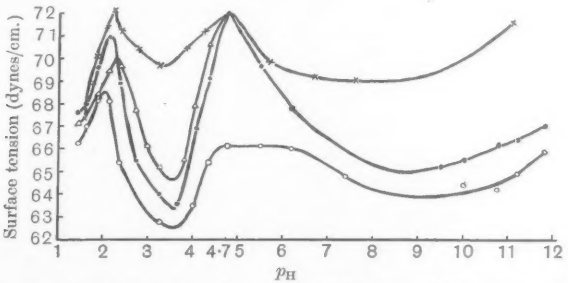

Fig. 2. Variation of surface tension with p_H of 0·4 % and 1·0 % gelatin solutions at 25° and 40°.

× 0·4 % gelatin in presence of HCl or NaOH at 25°.
● 1·0 % „ „ „ „ „ 25°.
○ 1·0 % „ „ „ „ „ 40°.
△ 1.0 % „ „ „ H_2SO_4 at 25°.

The possible hydrolysis of gelatin in acid solutions of p_H less than 2·2–2·0.

It will be seen that on the corrected surface tension-p_H curve of gelatin solutions the second maximum occurs at p_H 2·2–2·0, which is at a point considerably more acid than we previously found. Hence it is still more likely that the fall of surface tension in the most acid solutions after the second maximum is due to the hydrolysis of the gelatin by the strong acid. An attempt was made therefore to investigate this by following the change of surface tension and of the amount of nitrogen estimable by formaldehyde titration with time. It was hoped that it would be possible to correlate a decrease of surface tension with an increased formaldehyde titration and that the effect would be found only in solutions of p_H less than 2·2–2·0.

A quantity of gelatin was prepared in the usual manner and found to have an ash content of 0·1 % on the dry substance. The solutions contained 0·5 % gelatin and were brought to p_H 2·4, 2·2, 1·9 by addition of the appropriate quantities of HCl, and a fourth solution was made up at the isoelectric point,

p_H 4·7. All the solutions contained 2 cc. saturated solution of thymol and a little toluene per 500 cc. as a preservative. The two solutions at p_H 4·7 and 2·4 are both alkaline to the maximum and hence should not show an appreciable alteration of formaldehyde-N or surface tension with time; the solution at p_H 2·2 is at the maximum, and that at p_H 1·9 is acid to the maximum. The solutions were kept in a constant temperature oven at 25°.

The total N of each of the 0·5 % solutions was found by Kjeldahl's method to be 0·0896 g. per 100 cc., from which the value for the concentration is calculated to be 0·498 % (taking gelatin to contain 18 % N). The formaldehyde-N is expressed as a percentage of the total N.

Only 50 cc. of gelatin could be spared for each estimation, so that small titres were obtained on which the error was 5–7 %.

At stated intervals a sample was withdrawn from the solution in the oven for the surface tension and formaldehyde-N determinations and any portion of the sample remaining was thrown away.

The results are shown in Table II.

Table II. 0·5 % *gelatin solutions.*

	p_H 4·7		p_H 2·4		p_H 2·2		p_H 1·9	
Days	% formaldehyde-N	Surface tension (dynes/cm.)	% formaldehyde-N	Surface tension (dynes/cm.)	% formaldehyde-N	Surface tension (dynes/cm.)	% formaldehyde-N	Surface tension (dynes/cm.)
2	2·5	67·5	2·8	66·9	2·8	69·6	3·5	68·5
8	2·8	—	2·8	66·6	3·1	68·4	4·4	—
10	—	67·0	—	—	—	—	—	64·8
16	2·8	—	2·8	66·9	2·8	67·8	3·1	66·8
23	—	—	—	—	—	68·5	—	68·0
27	2·5	68·0	2·8	66·8	2·5	—	3·1	—
42	3·1	66·2	3·1	66·8	2·8	65·4	3·7	66·7

The figures for formaldehyde-N at the commencement of the experiment were all higher than expected, especially when no acid was present, and, as the surface tensions were lower than we had previously found them, it was thought that the gelatin, which was from an old stock and had developed a slightly "gluey" odour, might be at fault. Further, the isoelectric solution never flocculated properly and the centrifuged filtrate from it contained 46 mg. N per 100 cc., a quantity much in excess of the 15–20 mg. per 100 cc. found by Knaggs, Manning and Schryver [1923]. The experiment was, therefore, repeated with some fresh gelatin from the same source—Nelson's Gold Label. After washing in the usual manner it was found to contain 0·06 % ash on the dry substance. A 1 % isoelectric solution flocculated completely in 2 days, giving a surface tension of 72·3 dynes per cm. The solutions were made up to a final concentration of 1 % and the p_H brought to 2·5, 2·1, 1·8, 1·4 by the addition of the appropriate amounts of HCl. The total N of each solution was found to be 0·179 %, of which 2·6 % was found to be formaldehyde-N, *i.e.* the same percentage as was found in the previous experiments with a

supposedly deteriorated gelatin. Toluene only was used as a preservative, as it was possible that the thymol had lowered the surface tension of the 0·5 % solutions. In this experiment the values of the surface tensions agree with earlier determinations, but owing to the abolition of the thymol slight mould contamination was found in the two less acid solutions after 4 months. The solutions were kept at 25°.

The results are shown in Table III.

Table III. 1 % *gelatin solutions.*

	p_H 2·5		p_H 2·1		p_H 1·8		p_H 1·4	
Days	% formaldehyde-N	Surface tension (dynes/cm.)	% Formaldehyde-N	Surface tension (dynes/cm.)	% formaldehyde-N	Surface tension (dynes/cm.)	% formaldehyde-N	Surface tension (dynes/cm.)
1	2·5	69·0	2·6	70·7	2·6	67·7	2·8	—
4	—	69·0	—	68·2	—	67·3	—	66·9
11	3·7	—	3·8	66·9	3·6	67·3	3·4	67·6
18	3·4	67·3	3·5	64·8	3·7	66·4	3·9	65·7
22	—	66·4	—	—	—	—	—	—
25	3·4	—	3·8	—	3·4	—	3·6	64·4
114	3·4	64·7	3·9	64·6	4·6	63·2	6·2	63·6

There is a gradual fall of surface tension coupled with a rise in the amount of formaldehyde-N present, and both these effects increase in a fairly regular manner as the acidity of the solution is increased, indicating a slow hydrolysis taking place at all acidities, even at the isoelectric point, and increasing with the acidity (the most acid solutions in each case gave the most formaldehyde-N on standing).

The amount of formaldehyde-N in the 0·5 % solution increased very slightly in 6 weeks, though the solution of p_H 1·9 showed a sudden increase in the first 2 days, after which it behaved like the other solutions. In the 1 % solutions all rose in 10 days, but after 4 months only the two most acid ones had risen further. The surface tensions however show a continuous fall over the whole period of the experiment in each case. The p_H were found unaltered at the end of the experiment.

There is no evidence that hydrolysis of the gelatin molecule commences suddenly at the p_H of the second maximum of surface tension. Hence we are unable to ascribe the decrease in surface tension which we find in solutions of p_H less than 2·2–2·0 to hydrolysis with the formation of a less complex "acid protein" titratable in presence of formaldehyde.

Recent experiments by Sørensen [1925] on the coagulation of egg-albumin suggest another possible explanation. Sørensen found that the first process of coagulation is denaturation, which probably consists of the splitting-off of water from the protein, and that decomposition with formation of substances titratable in presence of formaldehyde is only a secondary process occurring on heating the denatured protein. The denaturation takes place more rapidly and at a lower temperature the higher the concentration of H or OH ions,

whilst the subsequent coagulation of the denatured protein, which behaves like a typical suspensoid in the case of egg-albumin, is most rapid at the isoelectric point. A similar denaturation is possibly occurring in the case of gelatin, the increase of formaldehyde-N in the most acid solutions on long standing at 25° being due to the secondary decomposition of the denatured gelatin.

Owing to the high solubility and non-coagulability on heating of gelatin, no definite evidence of denaturation of gelatin was obtained experimentally, but work now in progress on other proteins shows a relationship between the fall of surface tension with increasing acidity and denaturation of the protein.

Summary.

1. The "protein error" of certain indicators with gelatin solutions has been investigated. Bromophenol blue gives a minimum error at the isoelectric point and a maximum error about p_H 2·4.

2. The surface tension-p_H curves of gelatin have been corrected as regards p_H for the "protein error" in the colorimetric estimation of p_H.

3. The possibility of acid hydrolysis of gelatin in solutions acid to p_H 2·0 has been investigated by means of formaldehyde titration. A certain amount of hydrolysis occurred on long standing, but there is no evidence of an immediate hydrolysis which would account for the sudden fall of surface tension below p_H 2·0.

We are indebted to the Directors of Bass, Ratcliff and Gretton, Ltd., for permission to publish this work.

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CIV. THE NATURE OF THE SUGAR RESIDUE OF THE CEREBROSIDES OF OX-BRAIN.

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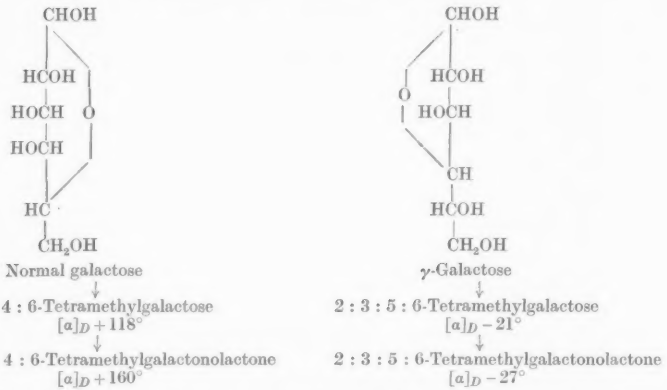
Since the publication of our preliminary note on the methylation of the cerebrosides of ox-brain [Pryde and Humphreys, 1924], investigations which have been pursued in this and in other laboratories have effected a considerable simplification in regard to the problem of the nature of the internal oxygen bridge in the sugars and their derivatives. Briefly, the present state of our information enables us to state that in the instances of the pentoses xylose and arabinose, the methylpentose rhamnose, and the hexoses galactose, mannose, glucose and fructose, the stable forms of these sugars possess an amylene oxidic bridge, whilst the reactive modifications synthesised by the action of cold acid methyl alcohol (the so-called "γ" forms) possess a butylene oxidic bridge. It seems highly probable that this generalisation will be extended to other hexoses and aldopentoses.

It was towards the solution of the nature of the oxidic linkage of the galactose residue of the cerebrosides that our investigation was directed, and the advances which have been made in the general chemistry of the sugars, and particularly in the instance of galactose, enable us to arrive at a decision with fewer experimental steps than were at first contemplated. In the case of this particular hexose our own results [Pryde, 1923; Pryde, Hirst and Humphreys, 1925], and those of Haworth, Ruell and Westgarth [1924], establish the fact that the butylene and amylene oxidic derivatives of galactose exhibit an opposition in the sign of their optical rotations, an observation which accords with the application of Hudson's rule to the stereochemical configuration assigned to galactose by Emil Fischer. The recent work of Levene and Sobotka [1926] on the isomeric tetracetyl derivatives of galactose is also in accord with these observations. The essential facts are summarised on p. 826.

In our previous communication [1924] we have shown that the cerebrosides undergo methylation by means of Purdie's reagents without suffering degradation, and that after subsequent treatment with acid methyl alcohol it is possible to isolate the methylated galactose residue. It is our experience, and also that of other workers in the sugar group, that the position of the oxygen bridge in glucosidic derivatives cannot change unless the glucosidic linkage is ruptured. Further, since methylation of the free hydroxyl groups preceded hydrolytic cleavage of the galactosidic linkage in the experiments



referred to above, it follows that the particular structural modification of the sugar as it occurs in the cerebrosides will be preserved in the form of methylated galactose finally isolated. Thus, if the oxygen bridge should be of the butylene oxidic type the final product would be 2 : 3 : 5 : 6-tetramethylgalactose, whilst if it should be of the amylene oxidic type, that is to say the normal, stable type, the final product would be 2 : 3 : 4 : 6-tetramethylgalactose. From the data given above it is clear that these two isomeric methylated galactoses may be readily distinguished from one another, since the former substituted sugar has a laevo-rotation, whereas the latter is dextrorotatory. It is therefore possible definitely to characterise the nature of the galactose residue by a determination of the optical rotation of the sugar obtained on scission of the methylated cerebrosides.

The mixed cerebrosides obtained from ox-brain by Rosenheim's method [1913] were subjected to a series of methylations with silver oxide and methyl iodide. Previous to methylation no attempt was made to separate the cerebroside mixture into phrenosin and kerasin owing to the great technical difficulties involved in their fractionation. Moreover, there appeared to be no reason for assuming that a homogeneous sugar residue should not be obtained from the methylated mixture. In its initial stages the methylation of the mixed cerebrosides proved to be as troublesome as that of the individual pure cerebrosides used in our earlier experiments. Nevertheless, after two methylations the further use of an extraneous solvent was unnecessary, whilst an alcohol-chloroform mixture (1 : 2) proved very efficient as an extractive solvent. After five repetitions of the methylation process a fully methylated product was obtained ($OCH_3 = 19{\cdot}04\ \%$), and after hydrolysis with acid methyl alcohol at 100°, the product, freed from solvent, was fractionally distilled in a high vacuum and thus the tetramethyl-methylgalactoside, formed by simultaneous hydrolysis and condensation, was separated from the fatty acid ester and the sphingosine which remained behind in the still. On hydrolysing this tetramethyl-methylgalactoside to the corresponding tetramethylgalactose a highly dextro-rotatory sugar was obtained, having an equilibrium value of

$[\alpha]_D + 115°$ in water. This value is but slightly below that of the purest preparations of amylene oxidic tetramethylgalactose so far recorded ($[\alpha]_D + 118°$), and allowing for the presence of a small amount of degradation products carried over with the sugar during the distillation, it affords conclusive proof of the homogeneous nature of the sugar residue of the cerebrosides. This observation therefore constitutes the first conclusive proof that the oxygen bridge of a naturally occurring galactoside, in which galactose comprises the sole carbohydrate residue, is of the same amylene oxidic type as that of the normal, stable sugar. The galactosidic residue of the cerebrosides is therefore similar to the sugar residue of all the naturally occurring glucosides so far examined, since the latter have been proved to contain the stable or amylene oxidic type of glucose[1].

Experimental.

For the preparation of the mixed cerebrosides used in this investigation we have used Rosenheim's method [1913]. The product obtained by pyridine extraction followed by precipitation with acetone was freed from traces of phosphatides by extraction with ether in a Soxhlet apparatus. The slightly yellowish cerebrosides thus obtained were recrystallised four times from 15 volumes of an alcohol-chloroform mixture (1 : 2). The final product as used by us was a fine, practically white powder, some 20 g. being obtained from 1 kg. of ox-brain.

Methylation of the mixed cerebrosides.

A typical experiment, in which 10 g. of the above product were used, is described. Methyl alcohol was used as an extraneous solvent during the first two methylations. The partially methylated product was then sufficiently soluble in methyl iodide to render the use of an extraneous solvent unnecessary. The course of the methylation is summarised in the following table:

Weight of cerebrosides used = 10 g.

No. of methylation	Reagents	Extraneous solvent	Extractive solvent	Methoxyl content	Melting point
1	30 cc. CH_3I 50 g. Ag_2O	30 cc. MeOH	EtOH + $CHCl_3$ (1 : 2) hot	—	—
2	25 cc. CH_3I 40 g. Ag_2O	10 cc. MeOH	EtOH + $CHCl_3$ (1 : 2) warm	8·75 %	75°
3	25 cc. CH_3I 30 g. Ag_2O	—	„ „	—	—
4	20 cc. CH_3I 20 g. Ag_2O	—	EtOH hot	18·6 %	35–40°
5	15 cc. CH_3I 10 g. Ag_2O	—	„	19·04 %	35–40°

Weight of methylated cerebrosides obtained = 9·56 g.
Pentamethylkerasin requires OCH_3 17·88 %.
Hexamethylphrenosin requires OCH_3 20·27 %.
A 50 % mixture requires OCH_3 19·08 %.

[1] In our previous communication [1924, p. 661] the statement is made that "all the naturally occurring glucosides so far examined have been shown to contain the stable 1 : 4 type of sugar." This statement was, of course, based on the assumption, generally accepted at that time [1924], that glucose in its normal stable configuration was butylene oxidic (1 : 4). Recently Charlton, Haworth and Peat [1926] and Hirst [1926] have demonstrated that normal glucose is amylene oxidic. The present authors have recently obtained independent evidence (unpublished) in support of this view. It therefore follows that all glucosides containing stable glucose are amylene oxidic.

Hydrolysis of the methylated cerebrosides and isolation of tetramethyl-methylgalactoside.

In continuation of the preceding preparation, 9·5 g. of the methylated cerebrosides were dissolved in 60 cc. of pure methyl alcohol containing 3 % of dry hydrogen chloride. The solution was heated in a sealed tube at 100° for 20 hours. On cooling in ice a gelatinous white precipitate separated and the solution became almost solid. The solution was filtered on a Hirsch funnel and the residue was washed with ice-cold methyl alcohol. The filtrate was then neutralised with silver carbonate and on removal of the solvent there were obtained about 4 g. of a brown syrupy residue. On extracting this residue with ether, the ethereal extract yielded about 2 g. of a dark yellow syrup, which was subjected to distillation in a high vacuum. The main fraction proved to be a colourless highly mobile liquid boiling at 125–130° at 1 mm. pressure. It had a refractive index of 1·4450 at 18·5° and a methoxyl content of 58 % in good agreement with that required for pentamethylgalactose.

Hydrolysis of tetramethyl-methylgalactoside.

The hydrolysis of the tetramethyl-methylgalactoside obtained from the methylated cerebrosides was effected by boiling with 8 % aqueous hydrochloric acid for 30 minutes. The hydrolysis was conducted in the presence of a little animal charcoal in order to adsorb the slight amount of coloured degradation products formed during the process. The methylated sugar was extracted from the neutralised, filtered solution by shaking with chloroform. After drying the solution over anhydrous magnesium sulphate the solvent was removed and the sugar was recovered in the form of a fairly viscous syrup. It was further purified by distillation in a high vacuum using a specially prepared micro-distillation flask. The final product proved to be a perfectly colourless syrup possessing all the characteristic physical and chemical properties of the equilibrium mixture of the α- and β-forms of amylene oxidic tetramethylgalactose. It had a methoxyl content of 51 %; $C_6H_8O_2(OCH_3)_4$ requires 52 %. Its rotation measured in aqueous solution ($c = 1{\cdot}0$ %) was $[\alpha]_D^{19^\circ} = +115^\circ$.

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CV. THE RESPIRATORY EXCHANGE OF THE MUSSEL (*MYTILUS EDULIS*, L.)

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(*Received July 12th, 1926.*)

Although much work has been done, notably by Vernon and Montuori, on the gaseous metabolism of marine and fresh-water invertebrates [*vide* summary given by Krogh, 1916], resulting in certain generalisations as to the influence of temperature, oxygen pressure and other physical factors upon the respiratory exchange, little or no work has been recorded bearing upon fluctuations of metabolic activity associated with the sexual and seasonal cycles. That such long-period fluctuations do exist has not, of course, been doubted, and the disparities between the results of different observers, or indeed of the same observer at different times, upon a given animal, have been attributed to physiological conditions, such as age, sex, or reproductive maturity, but no attempt has been made to give quantitative expression to these factors. The reason for this neglect is doubtless the fact that the difficulties inherent in the measurement of all biological responses are heightened in this case by the necessity of maintaining the organisms for long periods under controlled cultural conditions and by the uncertainty attached to the determination of age and maturity.

For the present investigation, an organism has been selected which combines with economic importance certain qualities which render it suitable for experimental work, such as hardiness under artificial conditions, ease of measurement and manipulation, and marked regularity of physiological rhythm. Individual variations have been reduced to a minimum by the use of large numbers of mussels in each experiment, and by statistical treatment of the results. The determinations, upon a carefully selected stock of mussels, were made at intervals throughout a complete reproductive cycle of one year, under conditions of temperature and oxygen pressure similar to those obtaining at the time. In this way, the mean respiratory values obtained during the course of the experiments, without any correction or "weighting," represent a close approximation to the sequence of metabolic changes as they occur in a natural state on the mussel-beds—a sequence of which every stage is the integrated response of the organism, as a living entity, to the ever-changing factor-complex of its environment. At the same time, by a statistical sorting-out process, the same experimental values have afforded data from which the

relative effects of the separate factors, both physical and physiological, have been calculated.

Certain aspects of the results here put forward stand in a most interesting relation to a study of the seasonal changes in the chemical composition of the mussel, carried out by Daniel [1921, 1922, 1923, 1925], and are discussed in detail on pp. 845 and 846.

Experimental.

The stock of mussels.

In order to ensure a reasonable uniformity of size and condition in the experimental material, a special stock was set aside at the outset of the work. This consisted of about 500 mussels, selected from a hundredweight taken from the beds at Ramsey, Isle of Man. By means of a simple "hit-and-miss" gauge, mussels between 5·0 and 6·0 cm. in length were alone selected, giving a stock of mean length 5·34 cm., and these were carefully scraped clean from any barnacles or other growths, and kept in a large shallow concrete tank of 120 litres capacity in one layer for the entire period of the work. Unfiltered water, pumped daily from the sea, was run through the tank continuously, at the rate of 240 litres per hour. No food of any kind was added to the water, but the sufficiency of the plankton which it contained was evident from the apparent well-being of the mussels and the great abundance of faecal matter, which was removed at intervals by completely emptying and brushing down the tank. The monthly mean temperature of the water varied between 7·5° in March and 15·5° in July; this range is very close to that of the coastal waters of the Irish Sea, which, for the 10-year period 1914–1923 was found to be 6·9° (March) to 14·7° (August.) The conditions under which the mussels were kept did not, therefore, differ widely from those obtaining on the mussel-beds, and even when mussels were removed from the tank for a respiratory determination, the experimental temperature was arranged to lie between the maximum and minimum, as then recorded, of the immediately preceding period. There was a slight mortality among the mussels amounting, at the end of the year, to about 11 % of the initial number, the deaths being spread very evenly throughout the period.

Variations in total wet weight, tissue weight, and shell weight, are recorded in the experimental series (p. 837); comparisons were made, from time to time, with mussels of the same length-range taken directly from the Ramsey beds, and in all cases the total weight, tissue weight and shell weight of the tank-material slightly exceeded those of the "wild" specimens. The length of the mussels (maximum length, with valves closed, as measured by a calliper gauge) increased during the year by 0·04 cm., or only 0·75 % of the initial mean length; there was, however, a considerable increase of shell weight. The entire weight of the individual mussels varied between 20 and 21 g., and each contained about 6 g. (wet weight) of living tissue.

Apparatus and method.

The apparatus devised for the respiratory determinations embodies several new features, and as it is suitable for a variety of aquatic organisms and is easy of manipulation, it will be described in detail. The respiratory chamber, *A* (Fig. 1), consists of a heavy glass vessel of about 6·5 litres capacity. The lid and lower portion, which are ground into smooth contact, are provided with flat flanges to facilitate clamping together when in use. Through a rubber stopper in the tubulure of the dome-shaped lid is fitted a telescopically arranged series of glass tubes, the innermost of which, *B*, reaching to the bottom of the vessel, and bent twice at right angles, forms a syphon outflow; the tube *C*,

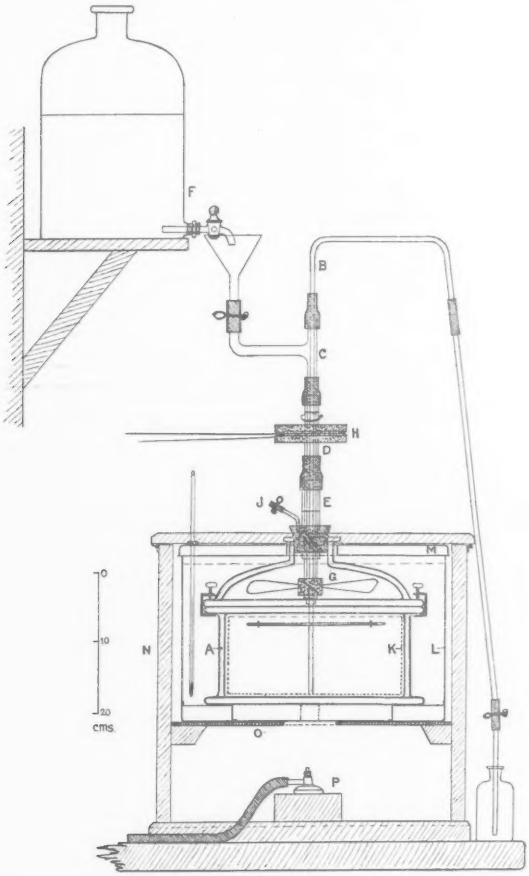

Fig. 1.

carrying a side branch and funnel at its upper end and reaching to the level of the lid-flange below, serves for filling the vessel or replenishing from the 10-litre reservoir, *F*, water withdrawn by the syphon, *B*; the outermost of the three tubes, *D*, carries a four-bladed ebonite stirrer, *G*, wholly contained within the dome of the lid, and a V-grooved pulley, *H*, whereby the stirrer is connected to a water-turbine or other motive arrangement. Other short lengths of tubing, *E*, associated with the central "telescope," act as bearings for the stirrer-shaft. Only one other tube, *J*, passes through the stopper. This ends flush with the underside of the stopper, and externally is fitted with a short length of pressure tubing and a spring clip; it serves as an air-escape, permitting the vessel to be completely filled with water; it is also temporarily unclipped when a sample is to be withdrawn by the syphon.

For convenience of handling, the mussels are not placed directly into the glass vessel, but into a special cage, *K*, made of perforated sheet-celluloid, which closely fits the respiratory chamber.

Such a large and heavy vessel cannot readily be placed in a thermostat of the ordinary type, but a very fair constancy of temperature is secured by having it supported, on radially projecting lugs, in a cylindrical galvanised iron tank, *L*, filled with water to the level of the broken line, and heated by a micro-burner, *P*, or cooled by ice, as the case may be. This tank has a loose drop-on lid, *M*, slotted for the tubulure of the respiratory vessel, perforated for the passage of a thermometer, and provided with two inspection holes 5 cm. in diameter. There is also a constant-level device, not shown in the figure, and a strong wire stirrup or handle, by means of which the respiratory vessel may be lifted from the tank. The tank itself is lagged with felt, to minimise heat-exchange, and the entire apparatus is mounted in a skeleton framework, *N*, of wood, and rests directly upon a "uralite" shelf, *O*, with a central perforation, to permit of heating, when required, by the burner in the space beneath. The two hinged strips of wood, forming the "lid" of the supporting framework, close tightly upon the cylindrical tank; they also carry upright rods, to which the funnel and central column are clamped when the apparatus is in use.

At the time of a respiratory determination, forty mussels are "selected at random" from the stock tank, and after a light scrubbing to remove diatoms or other surface growths and drying on a duster, their length, weight and volume are determined. The mussels are then placed in the perforated celluloid basket, which is lowered into the respiratory chamber, previously filled with sea-water. The apparatus is then assembled, the lid being luted on with rubber tap-grease and afterwards clamped, and the stirrer connected up to its motor. Water is also filled into the galvanised tank. Normally, the experiment is carried out at the temperature of the water in the stock-tank, and this is readily maintained by the water-jacket for several hours, since it is not likely to differ by more than one or two degrees from the air-temperature.

The mussels remain undisturbed for about half an hour, and then all the

water is run off, to be replaced immediately from the reservoir, the respiratory chamber being completely filled.

After five minutes, the stirrer is put into gentle rotation and the initial samples run from the syphon. The samples are collected in two stoppered bottles; one, of about 230 cc. capacity, for oxygen determination (p. 834), and the other, of 270 cc. and paraffin-coated, for determination of p_H and excess base (p. 835). These two samples, together with a small amount of water for rinsing the bottles, involve the removal of about 535 cc. from the respiratory vessel, or about 1/12th of the total volume, after deducting that occupied by the mussels and the celluloid basket. A corresponding volume of water is run in from the reservoir, the clips are closed, and respiration allowed to proceed for a predetermined period, usually 30 or 60 minutes, with occasional gentle stirring. At the end of the period, another pair of samples is withdrawn, and the volume again made up, each time noting the temperature in the vessel and in the water-jacket. The entire operation of removing samples and refilling occupies $2\frac{1}{2}$ minutes, and for this no allowance is thought to be necessary when recording consecutive results taken at, say, 30-minute intervals. Several determinations are made, as a rule, with progressively diminishing oxygen content; finally, samples are taken from the reservoir itself, in order that due allowance may be made, in each determination, for the added unused water.

At the close of an experiment the mussels are removed from the vessel and 20 of them taken for determination of tissue and shell weight. For this purpose, each mussel is prised open, care being taken not to injure the tissues, and the included or "mantle-cavity" water shaken out; after this, the valves of the shell are kept apart by means of a small wedge, and the mussels, placed on edge in a rack, are allowed to drain for an hour. The moist tissues are then scraped out into a tared beaker and weighed; the empty shells are weighed also, and both results expressed as mean weight per mussel. The remaining 20 mussels are replaced in the stock-tank. Since the weight of wet tissue per mussel varies during the course of the reproductive cycle, it has been necessary, in certain of the experimental series, to express the respiratory values in terms of a constant weight (10 g.) of wet tissue.

The condition of the tissues was always observed during the scraping-out process, and in view of recent work by Yonge [1925], special note was made of the appearance of the crystalline style. In no case was any difference noted between the styles of experimental and "wild" mussels, nor was there any correlation between the size and condition of the style and the reproductive phase of the animal. Although, as Yonge observes, the style may persist, in *Mytilus*, during starvation, it rapidly disappears when respiratory conditions are unfavourable. Other workers have, indeed, alleged that the style disappears during starvation, but it is probable that other factors, including oxygen-want, have contributed to this result. In any case, the present finding of the persistence of the style points to a fair normality of the experimental conditions.

Oxygen determination.

Winkler's original method, as applied to sea-water by Bjerrum [1904], was used throughout, with very slight modification. The amount of organic matter present was not sufficiently great to warrant the use of Winkler's later [1916] modification.

The sample of sea-water was drawn into a bottle of accurately known capacity (about 230 cc.) when full to the stopper. Immediately there were added, at the bottom, by means of a long-stemmed pipette, without mixing, 2 cc. of a saturated solution of nitrite-free KOH in 10 % KI solution and then 1 cc. of a 40 % solution of manganese chloride, containing 2 cc. of conc. HCl in each 100 cc. The stopper being inserted[1], the contents were mixed by rotation, when the whole of the dissolved oxygen combined with the precipitated manganese hydroxide.

$$Mn(OH)_2 + O \rightarrow H_2MnO_3.$$

After an interval, never less than an hour, when the precipitate had settled somewhat, an excess (3 cc.) of chlorine-free conc. HCl was run in at the bottom of the bottle and the contents thoroughly mixed.

$$H_2MnO_3 + 4HCl \rightarrow MnCl_2 + 2H_2O + Cl_2,$$

$$2KI + Cl_2 \rightarrow 2KCl + I_2.$$

The iodine set free was titrated (using 2 % soluble starch solution as indicator) with a standard solution of sodium thiosulphate, containing about 4 g. of the crystallised salt per litre. This solution was conveniently standardised against potassium bi-iodate in the presence of excess of KI and HCl.

It was found desirable to make up the thiosulphate solution a few weeks before it was actually required for use, and to check the standardisation occasionally or before each set of readings. The strength of the thiosulphate being known, the oxygen-equivalent is given by the relation:

$$24{\cdot}82 \text{ g. } Na_2S_2O_3, 5H_2O \rightarrow 0{\cdot}8 \text{ g. } O_2 \text{ or } 559{\cdot}8 \text{ cc. at N.T.P.}$$

All experimental data have been expressed, in the first instance, in terms of cc. of oxygen, measured as at N.T.P., used per mussel, or per given weight of mussel tissue, per hour. In this, as in all physiological work, however, it is the oxygen pressure, and not the absolute amount of the gas present, which determines its availability for respiratory purposes. The partial pressure exerted by the gas in solution equilibrium depends upon several factors, notably the solubility coefficient of the gas itself, the temperature of the solution, the prevailing atmospheric pressure and, in the case of sea-water, the concentration of dissolved salts. In the present investigation, since only freshly drawn sea-water has been used, of very constant and fairly high salinity (Cl = 18 ‰), the salinity factor may be regarded as a constant, and it has further been assumed that the differences in barometric pressure were never

[1] It will be obvious that 3 cc. of the sample spill over the neck, when this is done, but since the added reagents also contain oxygen in solution, no significant error is introduced.

sufficiently great to warrant any special correction being made on that account. Equilibrium, too, is slow of attainment, and the oxygen content of a water sample is not necessarily in equilibrium with the atmospheric or oxygen pressure prevailing at the time of its collection.

Fox's data [1907] were used in estimating the partial pressure exerted by the oxygen present, as determined by the Winkler method, at the observed experimental temperature.

Carbon dioxide determination.

The determination of CO_2 is unfortunately much less satisfactory than that of oxygen, and for this reason the discussion of the respiratory activity of the mussel, in relation to biological and physical factors, has been limited in this paper to data based upon oxygen determinations. At the same time, the carbon dioxide values have a certain relative significance and, when regarded in conjunction with the oxygen data, furnish an indication of the respiratory quotient and its variations. These latter values stand in such evident relation with the chemical changes of the mussel tissue as to afford a partial if indirect confirmation of their validity.

Direct gasometric estimation being impracticable, a method depending upon the determination of p_H by indicators, previously outlined by the author [Bruce, 1924], was adopted. As there stated, the method is not one of a high order of absolute accuracy, but under standard conditions is capable of giving useful results of relative significance, especially in serial experiments. The indicator employed was *m*-cresol purple, specially supplied by the British Drug Houses, Ltd., in "0·04 % solution in 20 % alcohol, half-neutralised with NaOH." This was found to give a satisfactory virage, easily read to 0·02 of a p_H unit, and presumed to have a "salt-error," at normal salinity, of 0·2 p_H, to be deducted from the apparent reading, when compared with the usual Clark and Lubs buffer solutions. The method of determination of excess base and full working details of the p_H method will be found in the paper referred to. No significant change in excess base was observed in any of the regular determinations, even after a specially prolonged experiment (18 hours), in which the mussels were confined in a small volume of water from which practically the whole of the dissolved oxygen was extracted, and which became so acid as p_H 7·4. This is of importance as indicating that the rise of CO_2 pressure, consequent upon continued respiration, was not so great as to bring into play the buffer mechanism whereby, in *Mya* and other forms, the calcium carbonate of the shell is dissolved in the restoration of the acid-base equilibrium.

The carbon dioxide results have been expressed in cc. of the gas evolved by a given weight of tissue per hour.

Results and Discussion.

As already indicated, determinations of respiratory activity were made at intervals, approximately monthly, throughout a year. On each occasion, the determination was carried out at several (up to eight) different oxygen pressures or temperatures, as the case might be, though a determination under normal (*i.e.* then obtaining) conditions of temperature and pressure invariably formed one of the series. In this way, a total of 43 readings was obtained. These were made on twelve different occasions, each reading representing the combined gaseous exchange of 40 mussels, for a period in no case less than 30 minutes. Despite the number and range of data thus made available, it was difficult, on account of the wide divergence between individual results, to discern on first inspection any general relationship between respiratory activity and physical or seasonal conditions. The inequality of the intervals between successive experiments, and the initial presumption that the smoothed results would not in any case lie upon a straight line, rendered it impracticable to apply the Method of Moments, and it was decided, therefore, to take averages of groups of results, plotting against a mean date the mean of the appropriate observations. In practice, the results obtained in any one month usually formed such a group, though occasionally any three successive observations have been averaged. This process was found to minimise random fluctuations, and gave results which, in graphic form, disclosed certain unmistakeable relations. It was felt that this method involved no great violence to the experimental values, especially since there was no evidence of sharp breaks in the plotted curves, and it was certainly justified in its result. It may be mentioned here that only one single experimental value was rejected in arriving at the means—a value so far anomalous as almost certainly to be the result of some untraced error.

The annual metabolic curve.

Observations were begun in March, 1925, and continued until April, 1926, spawning taking place at the end of May. As previously noted, 40 mussels were used for the respiratory determination, and 20 of these were subsequently taken for tissue and shell weight estimation. The former of these two values fluctuated rather widely, doubtless on account of differences in the rate of draining and drying which could not be eliminated. The successive determinations were for this reason averaged in groups of three, and are set out in Table I. Remarks upon the apparent condition of the mussels at the various dates, although made at the same time, are postponed to Table VI. The length measurements included in Table I are the means of several hundred individuals and are not subject to the group-averaging.

Table I. *Variations in total weight, tissue weight, shell weight and length, in* Mytilus.

Duration of Exp. (days)	Date	Mean length (cm.)	Mean entire weight (alive) wiped but not drained (g.)	Mean wet weight of tissues (g.)	Mean weight of shells (g.)	Mean weight of included water (by difference) (g.)
0	Mar. 5	5·34	—	—	—	—
25	Mar. 30	—	20·36	(7·20)	(10·16)	(3·01)
69	May 13	—	20·29	6·83	10·16	3·30
83	May 27	—	20·30	6·32	10·15	3·83
92	June 5	—	20·30	5·94	10·14	4·22
110	June 23	—	20·32	5·79	10·04	4·49
139	July 21	—	20·43	5·79	10·09	4·55
180	Aug. 31	—	20·57	5·83	10·60	4·14
196	Sept. 16	5·37	20·80	—	—	—
219	Oct. 8	—	21·04	5·78	10·72	4·54
267	Nov. 26	—	21·28	6·08	11·06	4·14
323	Jan. 22	—	21·02	6·04	10·87	4·11
370	Mar. 9	—	20·94	6·13	11·09	3·72
398	Apr. 7	5·38	21·02	—	—	—
408	Apr. 17	—	21·49	6·05	10·98	4·46

(Bracketed values are fair estimates.)

The above data show that the rapid loss of tissue weight from the 70th to the 90th day, consequent upon spawning, is made up for, in the total weight of the living mussel, by a corresponding increase in the included or so-called "mantle-cavity" water. There is a slow and only partial recovery of tissue weight, throughout the months following the spawning period, but the most striking feature is the rise in shell weight which, commencing in July, about the 140th day, continues until the end of November, when a constant weight is maintained. This gain in shell weight is not accompanied by any significant increase in length (0·2 mm.), and while it can hardly be supposed to have much, if any, influence upon the gaseous metabolism, the fact is of interest in the light of recent work by Orton [1925], on the conditions for calcareous metabolism in oysters and other marine animals. In this work, Orton adduces facts which point, in the case of the native oyster, to "a controlling factor represented by a minimum temperature, below which shell is not or cannot be laid down, and above which shell-material may be produced automatically." Other factors affecting shell growth are salinity and the reproductive activity of the animal itself. In the present instance, it is worthy of note that shell growth is at a standstill throughout, and for some time after, the actual spawning period, and that the high temperature of July and August, coupled with the cessation of the demand for gonad-material, has led to a renewal of shell deposition. The mere "relaying" of the mussels seems hardly adequate to account for the increase of shell substance, especially since it was not evident until five months after the mussels were subjected to the change of habitat.

The experimental temperature was never different by more than one or two degrees from that obtaining in the stock-tank in which the mussels were kept, and the variations of temperature in the tank itself followed closely

upon those in the coastal waters of the Irish Sea. Both stock-tank and experimental temperatures are given in Table II, as well as the "normals" for the Irish Sea, for the 10-year period 1914–23, which latter data are derived from the monthly weather reports of the Meteorological Office for those years.

Table II. *Monthly mean temperatures during course of mussel respiration experiment, compared with normal values.*

Month	Irish Sea (° C.)	Stock-tank (° C.)	Experimental vessel (° C.)
January	7·3	—	—
February	7·4	—	—
March	6·9	7·0	7·5
April	7·8	7·5	—
May	10·0	11·0	11·5
June	12·2	14·5	13·3
July	13·9	17·0	15·5
August	14·7	15·8	14·8
September	14·0	13·0	—
October	12·3	12·0	11·5
November	10·0	8·0	10·2
December	8·4	7·0	—
(January)	(7·3)	7·0	8·0
(February)	(7·4)	7·5	—

The oxygen content of the tank water was determined from time to time, consistently high values being observed. The health of the mussels, so far as could be judged from muscular tone and general appearance, remained normal, and there was no nett loss of tissue; on the contrary, there was a definite gain in shell weight, and spawning took place at the usual time. It may therefore be concluded that, as regards all the factors recognised as bearing upon the mussel's metabolism, the direct experimental values, smoothed by the monthly or ternary averaging, will afford a close parallel to the sequence of metabolic changes as they occur upon the mussel beds, in our coastal waters. This direct practical significance of the results must be emphasised; the animals were not narcotised or otherwise restrained, and while for this reason the experimental metabolic values may not strictly be regarded as "basal," it is certain that they approximate very closely to that standard. Muscular movements and slight locomotion usually occurred when the mussels were first placed in the respiration chamber, but all gross movement had ceased and the mussels were attached by their byssus-threads, before the initial period of accommodation had elapsed. During the actual experiment, the maintenance of the respiration current by ciliary action appeared to be the sole energy-requirement, apart from that involved in tissue-metabolism.

Table III embodies the complete series of monthly mean values. The respiratory exchange is expressed in cc. of oxygen (measured as at N.T.P.) absorbed *per mussel* per hour, and is not corrected in any way for temperature or other differences, as between successive members of the series. All the mussels were between 5·0 and 6·0 cm. in length. In arriving at these mean values, all experiments were disregarded in which the partial pressure of the dissolved oxygen, at the mid-period of the experiment, varied from the annual mean by more than 10 %. The actual oxygen uptake per 10 g. of wet tissue, and the

experimental temperature are also included, in order that the influence of varying body-weight and temperature upon the gross result may be judged.

Table III. *Seasonal variations in the oxygen uptake of the mussel*

(length range, 5·0–6·0 cm.), monthly means.

Date	Temperature (° C.)	Oxygen uptake per mussel per hour (cc. at N.T.P.)	Oxygen uptake per 10 g. tissue per hour (cc. at N.T.P.)	Number of experiments upon which mean is based
March 14	7·5	0·112	0·168	3
May 20	11·5	0·166	0·252	2
June 14	13·3	0·188	0·320	4
July 17	15·5	0·217	0·374	6
Aug. 31	14·8	0·161 (0·187)	0·276 (0·323)	1
Oct. 8	11·5	0·184	0·318	2
Nov. 26	10·2	0·116	0·191	4
Jan. 22	8·0	0·113 (0·114)	0·186 (0·182)	1
Annual means	11·5	0·157	0·261	—

(Figures in brackets are smoothed values, as used in the curves, to replace those based upon one observation only.)

The two sets of respiratory values, together with the corresponding temperatures, are plotted in Fig. 2, from which it will be seen that the temperature factor is the dominant one in determining the course of the metabolic rhythm. The relative importance of the several factors is dealt with more fully on p. 844. The loss of weight, consequent upon spawning, is obviously responsible for the increased disparity between the two respiratory curves, after the spawning period in May.

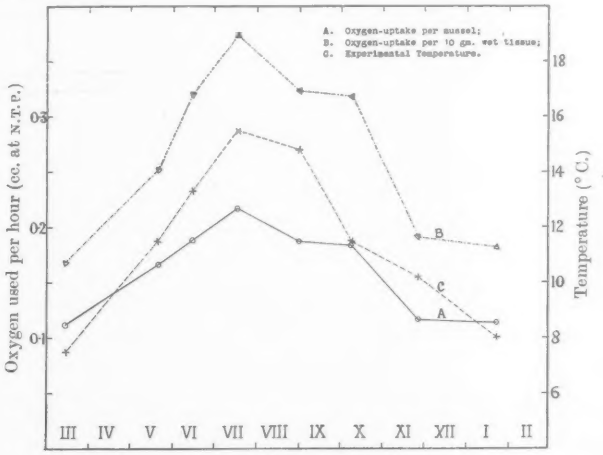

Fig. 2.

Effect of temperature on the gaseous metabolism.

In order to obtain a definite idea of the magnitude of the temperature effect, and a quantitative relation which would make it possible to reduce all

results to a common temperature, values were selected from the entire series of experimental data, which appeared to be least affected by conditions other than temperature. In making this selection, two criteria were observed.

(*a*) The values must lie within a narrow and normal range of oxygen pressure (say 20 mm.), so as to eliminate, as far as possible, variations due to that factor. The range actually tolerated was 133·3–154·0 mm. (The normal atmospheric pressure due to oxygen is 160·0 mm.)

(*b*) The values must not refer to physiological conditions of the mussels near the critical (spawning and fattening) periods. The period selected, being a flat region on the wet weight curve, was from June 23rd, 1925, to March 9th, 1926.

Data complying with these criteria are set out in Table IV, where the respiratory values refer, of course, to a constant weight (10 g.) of wet mussel tissue. The curve expressing these results is shown in Fig. 3, and it will at once be noted that the respiratory activity rises at a very regular rate with increasing temperature, the relation being practically one of direct proportionality over the range 3°–16°. At higher temperatures there is a falling-off, until, after passing through a maximum at about 22·5°, there is a rapid drop towards the thermal death-point, which was not, however, reached in this work, as the mussels kept for an hour at 25° and at 30°, though rendered flaccid, soon recovered, and in fact gave a normal respiratory value when tested at 15° some hours later.

Table IV. *Effect of temperature on the oxygen uptake of the mussel.*

Experimental values		Mean values	
Temperature (° C.)	Oxygen uptake per 10 g. wet tissue per hour (cc. at N.T.P.)	Temperature (° C.)	Oxygen uptake per 10 g. wet tissue per hour (cc. at N.T.P.)
3·5	0·022	3·5	0·022
5·5	0·095	5·8	0·098
6·0	0·100		
7·0	0·173	7·7	0·186
8·0	0·186		
8·0	0·198		
10·5	0·349	11·5	0·282
11·3	0·191		
11·5	0·320		
12·8	0·266		
15·0	0·472	16·2	0·430
16·5	0·448		
17·0	0·371		
22·3	0·549	22·3	0·549
25·0	0·480	25·0	0·480
30·0	0·166	30·0	0·166

From the respiration-temperature curve several interesting points may be deduced. The numerical value of Vernon's "temperature increment," defined by Krogh [1916, p. 89] as the ratio of the respiratory exchange at 24° to that at 10°, is seen to be 2·06, which is about normal for organisms of this type, hyaline forms giving a higher value. Since direct calorimetric measurement

of the respiratory energy is difficult, or impossible, in the case of slowly metabolising aquatic organisms, the calorific equivalent of the oxygen absorption could only be arrived at indirectly. On the usually accepted basis of 1 litre of oxygen being equivalent to 4·8 Cal., the oxygen uptake of the mussel-tissue, at 14°, should give rise to 0·178 Cal. per kilogram per hour. Respiratory determinations carried out by Jolyet and Regnard [1877] upon a large number of aquatic animals gave, in the case of *Mytilus*, at 14°, the value 0·06 Cal. per kilogram per hour, when the entire weight of the living animal, including the shell and presumably the included water, was used in the computation. The results obtained in the present investigation, when recalculated on this somewhat irrational basis, give a value of 0·051 Cal.—a close agreement, when it is remembered that the earlier work was based on eudiometric measurement of the extracted gases, and a totally different technique.

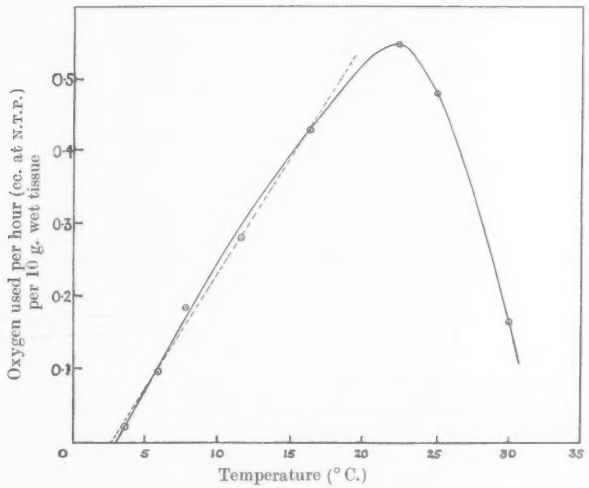

Fig. 3.

Finally, it is possible, by considering only that portion of the curve which most nearly approximates to a straight line, to obtain a temperature coefficient of the rate of oxygen uptake. The relation

$$t = 31r + 2{\cdot}6$$

(where t = temperature in ° C.,

r = respiration-rate (cc. of O_2 per hour per 10 g. wet tissue)

determines a straight line, and from this equation it is easy to reduce the respiratory values lying within its range (3°–16°) to any desired common temperature. As a matter of fact, 10° was chosen as the standard, since in addition to its general convenience, it happens to be the mean annual temperature of the Irish Sea coastal waters (Table II).

Effect of oxygen pressure on the gaseous metabolism.

Previous observers are in general agreement that marine invertebrates are remarkably insensitive to oxygen pressure, and that the respiratory rate remains undiminished until quite low oxygen pressures, say 30 % of the normal, are reached. The results given in this paper, while bearing out the general view that oxygen pressure is a factor of minor importance, point to the conclusion that the respiratory rate is slightly and evenly affected over a range of oxygen pressures extending far below and far above those normally experienced.

Table V. *Effect of oxygen pressure upon the oxygen uptake of the mussel at* 10°.

Range of partial pressure exerted by dissolved O_2 (mm. of Hg)	Mean oxygen uptake (corrected to 10°) per 10 g. wet tissue per hour (cc. at N.T.P.)	Number of experiments upon which mean value is based
80–99	0·189	3
100–119	0·184	5
120–139	0·220	7
140–159	0·226	9
—	—	—
390–415	0·450	4

In order to secure a sufficiently wide range of data it was necessary to accept the validity of the temperature correction arrived at in the preceding section, and the experimental values, corrected thus to 10°, were grouped in ranges of 20 mm. oxygen pressure, the mean results being listed in Table V.

These values, though slightly irregular, may be regarded as determining a straight line, of which the equation is:

$$p = 1165r - 120$$

(where p = partial pressure of O_2, in mm., of Hg,
r = respiratory rate (cc. of oxygen per hour, per 10 g. wet tissue), corrected to 10°).

As this line cuts the respiration ordinate at a positive value at zero oxygen pressure, it follows that it does not hold good down to the lowest pressures. It is valid, however, over the range experimentally realised (90–400 mm.), and provides a means, as in the case of the temperature curve, for reducing the readings to yet another common basis. In this case, 160 mm. is selected as the standard oxygen pressure, since that is the partial pressure exerted by the 21 % of oxygen in a normal atmosphere of 760 mm. At the same time, it is to be observed that complete saturation of the sea-water with oxygen is only occasionally realised, and that a mean annual oxygen pressure of 140 mm., or even lower in areas contaminated by sewage is likely to occur over the mussel beds.

A certain reservation must be made when interpreting the foregoing results, for although the supernormal oxygen pressures were arrived at by saturating sea-water with the gas under the pressure of a column of mercury, the

diminished or subnormal pressures were brought about, in each case, by the metabolic activities of the animals themselves. The low oxygen pressures were therefore inevitably accompanied by an increased concentration of dissolved carbon dioxide, and of hydrogen ions, and possibly also of excretory organic matter. That these factors may exert an effect upon the respiration rate, independent of that due to low oxygen pressure, is not improbable, though a careful examination of the results, corrected for both temperature and oxygen pressure, failed to reveal any significant dependence upon p_H over the range 8·15–8·55, and it may be supposed that any effect attributed to carbon dioxide is mainly brought about by the collateral change in hydrogen ion concentration. The effect, if any, of the presence of organic matter in solution was not determined.

The reproductive cycle.

It will be convenient to group under the above heading those factors of a physiological character which remain to influence the curve of respiratory exchange, after the effects of temperature and oxygen pressure have been neutralised by the appropriate correction formulae. For this purpose the combined results of each experiment are available, since it is desired to follow the effect, if any, of the reproductive factor throughout the year. The corrected results are still erratic, and in Table VI they are given, as averaged in groups of three.

Table VI. *Effect of reproductive activity on the oxygen uptake of the mussel at* 10° *and* 160 *mm. oxygen pressure.*

Date	Mean oxygen uptake (corrected to 10° and 160 mm. O_2 pressure), per 10 g. wet tissue per hour (cc. at N.T.P.)	Remarks on condition, etc.
Mar. 30	0·276	Acclimatised 30 days in stock-tank; good condition
Apr. 17	0·288	All tissues full of spawny patches
May 13	0·241	" "
May 27	0·247	Spawn running freely
June 5	0·233	Spawning apparently finished
June 23	0·251	A few patches of spawny tissue remaining in some mussels
July 13	0·217	" " " "
July 21	0·207	Tissues thin and "watery"
Aug. 31	0·215	" "
Oct. 8	0·234	Small patches of spawn in the mantle
Nov. 26	0·262	Considerable development of small patches of spawn in the mantle
Jan. 22	0·241	Considerable development of small patches of spawn in the mantle
Mar. 9	0·289	Mantle full of spawny patches

These results are shown in graphic form in Fig. 4. It is evident that the period from July to March, when the reproductive reserves are presumably being built up, is marked by a 30 % increase in the oxygen uptake per unit weight of wet tissue, though this is only apparent when the readings, made on a falling temperature gradient, are reduced, as here, to a common basis of 10°. Since the unit of wet weight (10 g.) must contain, at the successive

dates, an ever-growing proportion of gonad material, it would appear that those tissues of the mussel which are impregnated with the sexual products make a higher oxygen demand, weight for weight, than when they are in a resting or non-fertile state. Daniel [1925] adduces evidence to show that even when deprived of all particulate food for a lengthy period, the mussel does not draw upon the extensive reserves of glycogen and fats destined for the up-building of the reproductive products. In the starving mammal, as is well known, the carbohydrate reserves of the liver are mobilised at an early stage, and even in the mussel it can no longer be doubted that the glycogen, with which the tissues are replete at certain seasons, or at any rate its soluble derivatives, enter into the oxidative reactions which find expression in the respiratory exchange. It is the more surprising, therefore, to find that these stores of potentially valuable reserve materials should not be available for any but the sexual activities. The point is discussed in the ensuing section.

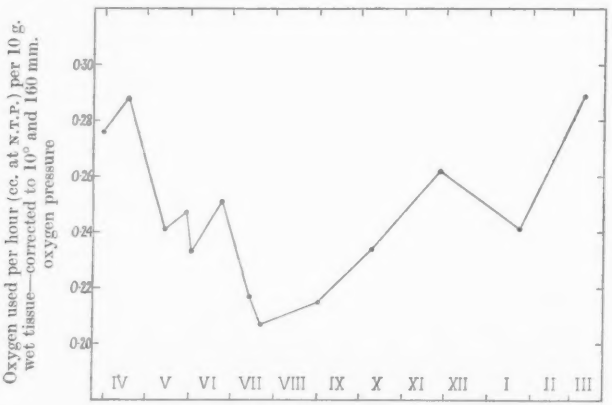

Fig. 4.

It will be convenient at this juncture to compare the relative effects upon the gaseous metabolism of the several factors—temperature, oxygen pressure and reproductive activity—so far considered. The range of monthly mean temperature experienced in the course of the year by mussels in their natural habitat may be taken as about 8°, with a minimum temperature of 6·9° in March, and a maximum of 14·7° in August (Table II). Other things being equal, this should lead to a range of oxygen uptake (per 10 g. wet tissue per hour), of 0·24 cc., the extreme values being 0·145 cc. in March and 0·385 cc. in August, as read from the curve in Fig. 3. Variations in sexual maturity throughout the year give rise to a range of only 0·08 cc., the lowest value, 0·21 cc., occurring in July, and the highest, 0·29 cc., in March (Fig. 4). It will be seen, therefore, that the fluctuations of temperature during a normal year may give rise to a

metabolic reaction three times as great as that due to innate physiological causes in the same period. The maxima and minima of these two factors, respectively, are almost coincident in time, and this tends, of course, to a less marked oscillation of the curve of nett metabolism (Fig. 2) than would be the case if they varied in true "phase" with each other. This curve only holds good for the Manx mussel under consideration, and will be modified in the case of mussel races such as those of the Irish and Lancashire coasts which spawn at other times of the year. Variations due to oxygen pressure, in so far as that factor is dependent upon the total barometric pressure, are still smaller, the range being of the order of 0·03 cc. of oxygen used for the mean annual barometric range. The pressure factor, moreover, is naturally irregular, and not seasonal, in its incidence.

The respiratory quotient.

The respiratory quotient not infrequently affords a valuable guide to the nature of the substances primarily involved in the respiratory oxidations, and for this reason determinations of the carbon dioxide output, as well as of the oxygen intake, were made in the manner already described. The method of CO_2 determination is admittedly not of a high order of accuracy, but the results obtained, and the corresponding respiratory quotients, are relatively valid, though the numerical scale must be regarded as arbitrary. The values of the R.Q. set out in Table VII are in each case the means of several determinations made in each separate experiment, but they are not subject to any further group-averaging.

Table VII. *Variations in the respiratory quotient of the mussel.*

Date	Mean respiratory quotient (arbitrary units)	Date	Mean respiratory quotient (arbitrary units)
Mar. 30	0·95	July 13	0·59
Apr. 17	0·62	July 21	0·25
May 13	0·61	Aug. 31	0·45
May 27	0·65	Oct. 8	0·44
June 5	0·45	Nov. 26	1·22
June 23	0·53	Mar. 9	1·31

The trend of the values is unquestionably to be associated with the changing chemical composition of the tissues [Daniel, 1921, 1922]. The rising quotient, from July to March, coincides with the period when carbohydrate (glycogen) reserves are being laid down, preparatory to the rapid metabolic changes leading to fat formation and a correspondingly low respiratory quotient, from March onwards to the actual spawning period in May.

It is generally recognised that, in the mammalian economy, "different proteins, fats and sugars are not physiologically equivalent, but that certain definite chemical groups subserve special functions in the animal organism" [Dakin, 1922]. Here, in the mussel, is evidence of the same metabolic specialisation. The wide disparity between the spring and autumn values of the respiratory

quotient, and the variation of absolute oxygen uptake, which follows a very similar curve (Fig. 4), point to a profound difference of metabolism at these periods. With this difference the non-availability of the reproductive reserves for the ordinary activities of the body is almost certainly to be associated, though a full understanding of the metabolic relations can only be obtained when to the mere knowledge of the gaseous intake and output of the organism there is added an appreciation of the numerous reactions involved in the intermediary metabolism. This is a highly complex problem which awaits investigation.

Summary.

1. The gaseous metabolism of the common mussel (*Mytilus edulis*, L.) has been studied throughout an entire reproductive cycle.

2. The variations in the oxygen requirement of a full-grown mussel, under the factors, both physical and physiological, normally incident upon the mussel-beds in the course of a year, have been determined.

3. Quantitative expressions have been obtained for the effect of temperature and oxygen pressure upon the rate of oxygen uptake, which is also profoundly affected by the state of reproductive maturity. The influence of the annual temperature wave is approximately three times that attributable to the sexual cycle.

4. The seasonal changes in absolute oxygen requirement, and the respiratory quotient, are intimately associated with concurrent changes in the chemical composition of the tissues.

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CVI. POSSIBLE SIGNIFICANCE OF HEXOSE-PHOSPHORIC ESTERS IN OSSIFICATION.

PART VI. PHOSPHORIC ESTERS IN BLOOD-PLASMA.

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The explanation of the process of calcification suggested in the first of these papers requires that a suitable phosphoric ester shall be supplied to those regions of bone and cartilage where ossification is taking place. It has been shown that one of the acid-soluble phosphoric esters occurring in blood fulfils the conditions of suitability in that it yields a soluble calcium salt and is hydrolysed by aqueous extracts of the bone enzyme, but the fact that these esters are almost entirely confined to the corpuscles[1] makes it necessary to enquire further into the possibility of this compound passing into the fluids bathing the cartilage cells. This possibility would not be excluded even if the ester were normally absent from blood-plasma, for a varying permeability of cell membranes must be assumed to explain many of the facts of physiology.

The amounts of organic acid-soluble P found by previous workers in normal plasma or serum are set out in Table I.

Table I.

Author	Animal	Organic P in serum (mg. per 100 cc.) Lowest	Highest	Average
Greenwald* [1916]	Sheep	0·43	1·06	0·68
"	Pig	—	—	0·46
Feigl† [1917]	Human	0·20	0·90	0·51
Bloor [1918]	Men	0·13	1·30	0·54
"	Women	0	1·30	0·40
Jones and Nye‡ [1921]	Children	(−2·0)	2·0	0·44
McKellips, De Young and Bloor [1921]	Human adult	0·06	1·3	0·48
" "	Human infant	0·40	3·6	2·21
Buell [1923]	Dog	—	—	Negligible
Plass and Tompkins [1923]	Human maternal	0	1·3	0·73
" "	Human foetal	0·30	3·1	1·23
Stanford and Wheatley§ [1925, 2]	Human	—	—	None

* Greenwald found that this organic P was not readily dialysable.

† Feigl has also investigated the organic P content of human serum in many pathological conditions. [See Feigl, 1920.]

‡ In view of the large experimental error shown by these results no great importance can be attached to this average value.

§ In four out of five plasmas examined, Stanford and Wheatley [1925, 2] actually found "unknown P" amounting to 0·5–0·8 mg. per 100 cc., but this does not appear to have been acid-soluble. The probable error of their method, which they give as ±5 % [1925, 1] would largely mask the small amounts of P which might be present.

[1] Ether-soluble phosphoric esters (phospholipins) occur in considerable amount in both plasma and corpuscles but these are not hydrolysed by the bone phosphatase.

The accuracy with which the total P can be estimated is the decisive factor in this question, since the organic fraction can only be determined by difference (total P – inorganic P), and it was therefore necessary to reduce the experimental error as far as possible and to have precise knowledge of its limits before we could place any reliance on our own results.

Method.

In a previous paper [Martland and Robison, 1924] certain possible sources of error in the estimation of P by the Briggs method were pointed out and it may be of use to describe the exact procedure now employed after 2 years' further experience. It should be added that the slight modifications introduced involve no radical change in this very useful method. The following solutions are used:

A. Concentrated H_2SO_4 30 cc.
Water to 100 cc.

B. Ammonium molybdate 10 g.
Water to 100 cc.

C. Sodium sulphite 20 g.
Quinol 0·5 g.
Water to 100 cc.

The sulphuric acid and ammonium molybdate are now added separately in estimating both inorganic P and total P, in order that the same solutions may be used throughout. If the molybdate solution is kept for any length of time a precipitate may form and if the amount of this is large the solution should be rejected. For this reason the original Briggs solution is preferable for routine use when only inorganic P is to be estimated.

From plasma or serum the proteins are precipitated in a dilution of 1 in 5 with 3·5 % trichloroacetic acid. Whole blood or corpuscles are first laked with 1 % trichloroacetic acid and the proteins are then precipitated by raising the concentration of the acid to 3·5 %. Filtration is carried out after 15 minutes through a P-free filter paper (No. 30 Whatman), slight suction being permissible.

Inorganic P. 5 cc. of the filtrate (equivalent to 1 cc. plasma or serum) are pipetted into a 15 cc. graduated flask, a suitable volume of the standard KH_2PO_4 solution (1 cc. = 0·01 mg. P) being measured into a similar flask. Both the solutions are diluted to about 10 cc. with water and 1 cc. each of solutions A, B, C are then added, the flasks being gently shaken between each addition. After dilution to the mark the contents are thoroughly mixed and the colorimeter readings are taken after half an hour. It is advisable to calibrate the graduated flasks and to make a correction for the volume error, which is frequently considerable.

Total acid-soluble P. We have abandoned the use of the nitric-sulphuric acid mixture for ignition owing to the risk of low results due either to incomplete oxidation or, if a higher temperature be employed, to combination of

phosphoric acid with the glass. The latter can be easily demonstrated if a flask which has been etched in this manner is rinsed with water and then warmed with a solution of ammonium molybdate, ammonium nitrate and sulphuric acid as in the Neumann method for P estimation. The presence of phosphate is shown by the yellow precipitate formed on the etched surface. Such loss chiefly occurs through the overheating of those parts of the glass which are just above the liquid, but the danger is not entirely obviated by the use of narrow boiling tubes and small flames. Pyrex tubes heated in a metal bath below the boiling point of sulphuric acid have sometimes shown such etching.

The danger of incomplete oxidation at lower temperatures can be overcome by repeated ignitions with small additional amounts of nitric acid[1], but we have found Baumann's [1922] method of ignition with 30 % hydrogen peroxide and sulphuric acid both simpler and more satisfactory. With this agent oxidation is rapid and complete at such moderate temperatures as involve no risk of loss by etching. Baumann found it necessary to re-distil the hydrogen peroxide (Merck's "superoxol") which he employed. We have used Merck's "perhydrol" and up to the present have found it free from phosphorus. Other makers' preparations of similar strength have contained phosphorus in large amounts.

The procedure is as follows. 5 cc. of the trichloroacetic acid filtrate are measured into a Pyrex boiling tube; 1 cc. of solution A is added and the liquid is concentrated on the convenient electric heater described by Stanford and Wheatley [1925, 1] until slight charring takes place. 0·1 cc. of Merck's "perhydrol" is then added and the ignition continued for about 4 minutes, until the dense white fumes first evolved have cleared. The acid residue should be quite colourless, but if any charring persists a further drop of perhydrol should be added and the ignition repeated. When the tube has cooled the contents are rinsed with successive small quantities of water into a 15 cc. flask and 1 cc. each of solutions B and C are added[2], the standard being made up as before with all three solutions.

Factors affecting the accuracy of the method.

Proportionality of colour produced to phosphate present. We cannot agree with Stanford and Wheatley's [1925, 1] statements on the lack of proportionality between colour produced and phosphate present. We have no experience of Stanford's [1923] dilution colorimeter, but with an instrument of the Dubosc type (Bausch and Lomb) we find, as stated in our previous paper, that the intensity of the colour is proportional to the amount of phosphate within very wide limits. The following results may be quoted in support of this conclusion.

[1] The addition of sugar solution after ignition with nitric acid is not necessary. Such traces of nitric acid as remain do not affect the colour intensity.

[2] If the final ignition has been unduly shortened traces of peroxide (persulphuric acid) may remain and form yellow permolybdic acid on addition of solution B. This is reduced by the sulphite but may cause the results to be low.

Amounts of inorganic phosphate varying from 0·08 to 0·02 mg. P were compared with a standard containing 0·1 mg. P, the maximum difference corresponding with a ratio of 1 : 5.

The amounts of P calculated from the colorimeter readings are shown below:

mg. P present	·0800	·0600	·0400	·0200
mg. P found	·0805	·0603	·0400	·0196

It is obvious, however, that with the usual colorimeter scale the percentage accuracy of the readings will be greatest when both unknown and standard are of nearly the same intensity, and this will naturally be chosen as the optimum condition.

Effect of the degree of acidity on the colour intensity. We confirm our previous statement that although the rate of development of the colour depends on the degree of acidity, small variations (± 15 %) in the amount of acid, under the conditions given, have no significant effect on the colour produced in ½ hour to 2 hours. In particular, the presence of trichloroacetic acid in amounts up to 0·25 g. (*i.e.* a final concentration of 0·1 *N*) does not cause any inaccuracy in the result as is shown by the following estimations of inorganic phosphate.

Trichloroacetic acid added (cc. 25 % sol.)	0	0·5	1·0
mg. P present	0·0500	0·0500	0·0500
mg. P found	standard	0·0500 0·0506	0·0500 0·0499

The use of several standard solutions containing varying amounts of trichloroacetic acid, as advocated by Stanford and Wheatley, is thus an unnecessary complication.

Error of the method.

In routine estimations of inorganic phosphate where the amount present is about 0·05 mg. P we find that the error does not usually exceed ± 0·0005 mg. (± 1 %). By meticulous attention to every detail this error may be reduced by half. As in all colorimetric work, the possibility of occasional errors of relatively large dimensions must be reckoned with and these can only be guarded against by rigid controls.

The error of the method for total P was investigated by carrying out series of estimations on standard solutions both of KH_2PO_4 and of sodium glycerophosphate. In this way errors due to losses of P or other effects of the ignition were distinguished from such as might be due to incomplete oxidation of the ester. A large number of duplicate estimations of total P in blood filtrates has also been carried out. The common practice of determining the accuracy of a method by the recovery of added phosphate from blood or serum is unsatisfactory because it does not exclude the possibility of constant errors which may even be of large dimensions. We have frequently obtained duplicate results showing excellent agreement although wide of the correct value.

The standard glycerophosphate solution was prepared as follows. The

P contents of an approximately $M/30$ solution of sodium glycerophosphate and of a standard KH_2PO_4 solution (1 cc. = 1 mg. P) were estimated by Neumann's method, the mean of at least four concordant results being taken. Each solution was then diluted 1 in 100, using a standard pipette and flask. The exact value of the diluted glycerophosphate solution in terms of the diluted KH_2PO_4 solution was then known and the latter was used as standard in the colorimetric estimation.

We give a summary of the latest series of tests.

KH_2PO_4. Estimations carried out as for total P (perhydrol ignition).

Amount of P present. 0·1000 mg. No. of estimations 23. Average of all results 0·0995 mg. Extreme limits 0·1003 and 0·0986, but 19 results lay between 0·1000 and 0·0990 (± 0·5 % of average).

Sodium glycerophosphate.

(i) Amount of P present 0·1170 mg. No. of estimations 22. Average results 0·1165 mg. Extreme limits 0·1174 and 0·1154, but 17 results lay between 0·1171 and 0·1159 (± 0·5 % of average).

(ii) Amount of P present 0·0351 mg. No. of estimations 5. Average results 0·0348 mg. Extreme limits 0·0351 and 0·0347, but 4 results lay between 0·0348 and 0·0347 (± 0·3 % of average).

From these and many other tests we conclude that the results obtained by this method will probably be below the correct value by not more than 1 % and that at most the error should not exceed + 0·5 % to − 1·5 % of this value. Duplicate results on blood filtrates also fall within these limits.

Phosphorus in serum.

Having satisfied ourselves that we could determine the inorganic P and total acid-soluble P in 1 cc. of serum with sufficient accuracy to detect differences greater than 1 % we set out to examine blood taken from different animals under varying conditions so as to discover whether organic acid-soluble P actually occurs in the serum, and if so whether and under what conditions the amount varies.

No anti-coagulant was used as we wished to avoid any possible effect of such additions on the partition of the P compounds between corpuscles and plasma. The blood was centrifuged at once so that the corpuscles were thrown down before clotting began. The serum was separated by gentle pressure on the fibrin clot and again centrifuged. Sometimes it was possible to pipette off the plasma before the clot formed, but in any case it was centrifuged a second time to remove any residual corpuscles. The presence of 0·1 % of the red corpuscles—intact or laked—would account for about 0·04 mg. organic P per 100 cc. in human serum and 0·05–0·07 mg. in the case of rats or rabbits. Microscopical examination of the serum showed that the number of corpuscles remaining was always far below this figure, while the absence of haemolysis was shown by the colour and by spectroscopic examination. Haemolysis

corresponding with less than 0·05 % laked corpuscles would not, however, be easy to detect. We are satisfied that if any of the organic P is to be ascribed to such adventitious causes the amount cannot exceed 0·04 mg. per 100 cc. serum.

The possibility that the organic P was not of the acid-soluble type, but was due to traces of phospholipins which had escaped precipitation with the proteins, was also examined. 50 cc. of trichloroacetic acid filtrate (equivalent to 10 cc. serum) which contained 0·044 mg. organic P (0·44 mg. per 100 cc. serum) was extracted with ether. After distilling off the ether the residue was examined but was found to contain no P, thus confirming our previous observations on this point.

Tables II and III show the amounts of inorganic and organic P found in the serum of blood taken from normal subjects in the fasting and absorptive states, after the administration of large amounts of phosphates and after injection of certain hormones.

Table II. *Acid-soluble P in human serum.*

(Blood taken from arm vein in all cases)

Subject	Conditions of test	Acid-soluble P in serum (mg. P per 100 cc.) Inorganic	Organic	Organic as % of total
Man [R. R.]	After 17 hours' fast	3·42	0·34	9·0
	1½ hours after food	3·37	0·17	4·8
,,	After 21 hours' fast	3·81	0·24	5·9
	2 hours after food	3·74	0·25	6·3
,,	2 hours after food	2·50	0·25	9·1
	0·5 cc. pituitrin injected after the previous sample was taken, 2nd sample 50 minutes later	2·72	0·35	11·1
,,	2½ hours after food	3·58	0·18	4·8
	10 g. NaH_2PO_4 (+$NaHCO_3$) then taken in 1 % solution, 2nd sample 1 hour later	5·83	0·20	3·3
,,	30 g. NaH_2PO_4 (+$NaHCO_3$) taken in three doses at hourly intervals, blood withdrawn 1 hour after last dose	7·14	0·66	8·5
,,	25 g. NaH_2PO_4 (+$NaHCO_3$) taken in two doses before and after meal, blood withdrawn ¾ hour after last dose	5·49	0·35	6·0
,,	10 g. NaH_2PO_4 (+$NaHCO_3$) taken before meal, blood withdrawn 3½ hours later	3·91	0·04	1·0
Woman	After 13 hours' fast	3·82	1·27	25·0
,,	1½ hours after food	3·72	0·02	0·5
,, syphilitic	3 hours after food	3·80	0·14	3·6
,, ,,	2½ hours after food	3·76	0·16	4·1
,, ,,	1½ hours after food	4·26	0·13	3·0
,, diabetic	After 13 hours' fast	3·68	0·08	2·1
,, ,,	After 13 hours' fast	3·81	0·05	1·3

It must therefore be recognised that organic acid-soluble compounds of phosphorus are normal constituents of blood-serum and therefore of blood-plasma. The values here shown are probably too low on two accounts.

(1) The error in the estimation of total P already referred to. The average correction under this heading might amount to 1 % of the total P, although it would not be justifiable to apply this correction to any individual result.

(2) The hydrolysis of the phosphoric ester during the interval (40–50 minutes) between the withdrawal of the blood and the precipitation of the serum proteins. Experiments described below show that traces of phosphatase are present in serum and that as much as 0·1 mg. organic P might be hydrolysed in this time.

Table III. *Acid-soluble P in rabbit serum.*

Age of animal	Type of blood and conditions of test	Acid-soluble P in serum (mg. P per 100 cc.) Inorganic	Organic	Organic as % of total
3 weeks	Venous (ear vein)	11·20	0·28	2·4
4 ,,	Mixed	7·96	0·13	1·6
6 ,,	Arterial (cardiac puncture)	7·00	0·20	2·8
Adult	Mixed	4·32	0·30	6·5
,,	,,	8·52	0·44	4·9
,,	,,	3·18	0·31	8·8
,,	Arterial (cardiac puncture)	5·60	0·26	4·4
,,	Venous (ear vein)	4·53	0·42	8·5
,,	Venous (ear vein) 1st sample taken after feeding	7·94	0·36	4·3
,,	Animal then allowed to drink a solution of Na_2HPO_4 (0·35 g. P) + 20 g. glucose. 2nd sample taken 2½ hours later	7·91	0·48	5·7
,,	Repetition of above exp. 1st sample before taking Na_2HPO_4, etc.	7·97	0·29	3·6
	2nd sample after taking Na_2HPO_4, etc.	7·86	0·43	5·5
,,	Venous (ear vein). 1st sample taken after feeding	4·73	0·12	2·5
	Animal then given intramuscular injection of 0·5 cc. pituitrin (P.D.). 2nd sample taken 50 minutes later	5·68	0·22	3·7
,,	Repetition of above exp. but with two injections of 0·5 cc. pituitrin at an interval of 1 hour:			
	1st sample before injections	4·15	0·29	6·5
	2nd sample 15 minutes after 2nd injection	2·99	0·34	10·2

We conclude that the amounts of organic acid-soluble P present in the circulating plasma would probably be at least 0·1 mg. more than the values shown in the tables.

It is clear that the amount is by no means constant. In one subject (R. R.) 0·04–0·66 mg. per 100 cc., representing from 1 % to 11 % of the total acid-soluble P was found at different times, while as much as 1·27 mg. or 25 % of the total P was found in another case. Serum obtained from this subject on a later day contained only 0·02 mg., or 0·5 % of the total P, in organic form. The results do not, however, throw much light on the cause of these variations and we do not feel justified in forming any opinion on this question until further work has been done.

Experiments carried out with whole blood *in vitro* have shown that during the hydrolysis or synthesis of phosphoric esters which can be brought about by slight variations in the p_H of the blood [Martland, 1925], the changes in the amount of inorganic and organic P occur primarily in the corpuscles, and that the concentration of inorganic phosphate in the plasma is only slightly altered. The corpuscular membrane seems to be not readily permeable to inorganic phosphate ions and considerable difference in concentration may

thus ensue. This is probably the explanation of the different conclusions which have been formed as to the relative amounts of inorganic phosphate in corpuscles and plasma. The phosphoric ester, or esters, concerned in these reactions belong mainly to the fraction which is not hydrolysed by the bone enzyme [Martland, Hansman and Robison, 1924]. After many experiments, no satisfactory evidence has been obtained of any increase in the amount of organic P in the plasma during these changes which are brought about by the activity of enzymes in the corpuscles, nor have we any evidence of synthesis occurring in serum by the action of enzymes there present.

Hydrolysis of the phosphoric esters by the plasma phosphatase.

Blood-plasma contains small amounts of a phosphatase similar to that found in bone. Like the latter it hydrolyses sodium glycerophosphate, hexosemonophosphate and hexosediphosphate and has an optimum p_H in the region of 9, whereas the optimum for the corpuscle phosphatase is about 6. At the optimum p_H and in presence of excess of glycerophosphate or hexosemonophosphate serum of normal adult rabbits has been found to hydrolyse about 30 mg. P in 18 hours at 38°. The serum of a young rachitic animal showed a much greater activity (94 mg. P in 18 hours at p_H 8·4)[1].

This serum phosphatase was found to hydrolyse the organic P compounds of the serum itself, and although its activity at room temperature and at the p_H of the blood would be only a small fraction of its optimum, yet we have obtained evidence of small but definite hydrolysis of organic P during the time between the withdrawal of the blood and the precipitation of the serum protein. This loss may amount to about 0·1 mg. P as is shown by the following figures (mg. P per 100 cc.).

		Time after withdrawal of blood			
		Room temperature 50 minutes	Room temperature 1 hour 50 minutes	Room temperature 50 minutes + 3 hours at 38°	Amount hydrolysed
Serum 1	Inorganic P	3·41	3·53	—	—
	Organic P	0·32	0·22	—	0·10
Serum 2	Inorganic P	8·52	—	8·84	—
	Organic P	0·44	—	0·09	0·35

Action of the bone enzyme on the phosphoric esters in serum.

Human blood contains an average of about 20 mg. P per 100 cc. in the form of acid-soluble esters, of which about 6 mg. or 30 % is hydrolysed by the bone enzyme. The figures for rabbit blood are about 50 % higher in each case. The esters in the plasma might belong to either of these types or might be a mixture as in the corpuscles. The action of the bone extracts on these esters had been tested from time to time and the evidence seemed to show that the major portion was hydrolysed, but until recently these experiments did not

[1] Demuth [1925] has investigated the activity and optimum p_H of the serum phosphatase in various pathological conditions, using hexosediphosphate as substrate.

seem to us convincing on account of the small amounts of P involved, in comparison with the possible errors of estimation.

The following experiment carried out with all the precautions referred to above has justified the previous conclusion and shows that the whole of the organic P in this serum was hydrolysed in $3\frac{1}{2}$ hours at 38° by the bone phosphatase.

The trichloroacetic acid filtrate, obtained as described above from the serum of an adult rabbit, was neutralised with sodium hydroxide.

30 cc. were treated with 0·5 cc. of a 20 % extract of young rabbit bones, the p_H being adjusted to 8·6. 10 cc. were removed immediately for estimation of total and inorganic P, and the remainder was kept at 38°, 10 cc. being removed after $1\frac{1}{2}$ and $3\frac{1}{2}$ hours. The P in the bone extract was separately estimated and was equivalent to only 0·68 mg. P per 100 cc. serum. This has been subtracted from the results which are shown below.

	0 hours	$1\frac{1}{2}$ hours	$3\frac{1}{2}$ hours
Total P	8·97	9·01	8·97
Inorganic P	8·58	8·89	9·06
Organic P	0·39	0·12	—

The whole of the organic P in the serum amounting to 0·39 mg. per 100 cc. was thus hydrolysed in $3\frac{1}{2}$ hours under these conditions.

The partition of the hydrolysable ester between corpuscles and plasma would appear in this case to be roughly in the ratio of 25 to 1.

Summary.

The presence of small amounts of acid-soluble phosphoric esters in blood-plasma has been confirmed.

These esters are hydrolysable by the bone phosphatase.

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CVII. THE FORMATION OF POLYSACCHARIDES BY YEAST PREPARATIONS.

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It has been well known for a long time that a polysaccharide is synthesised from sugar by the enzymic action of micro-organisms. Cremer [1899] described how the fermentation of sugar by yeast-juice, which did not give a glycogen reaction with iodine, formed a new substance which gave a glycogen reaction. Harden and Young [1913] observed the production of dextrorotatory polysaccharides when glucose and fructose were fermented with Lebedev's maceration extract of dried yeast. In one case a substance was isolated, the solution of which was opalescent and gave a red coloration with iodine. Later Grey [1918] observed the formation of a non-reducing carbohydrate in the early stage of the fermentation of sugar by *B. coli* and he subsequently stated [1924] that starch giving the characteristic blue coloration with iodine was synthesised from sugar in the early stage of fermentation in the presence of chalk by an organism of the coli group.

Smedley-MacLean and Hoffert [1923] found that the presence of added phosphate slightly diminished the formation of polysaccharides in living yeast suspended in sugar solution, both in the presence and absence of oxygen.

The subject has acquired great interest owing to the recent investigations on the regeneration of glycogen in muscle, and the present work was undertaken in the hope (which was not fulfilled) of getting some knowledge of the mechanism of this change and particularly of the part, if any, played by phosphate in it. For this purpose experiments were made on the effect of addition of phosphate on the formation of polysaccharides from sugar by yeast-juice, maceration extract and zymin (yeast treated with acetone).

Experimental method.

In order to ascertain whether polysaccharides had or had not been formed the following method was adopted. The sample under examination was diluted to a known bulk and at the same time sufficient N HCl was added to make the liquid $N/10$. In this way the fermentation was stopped and the precipitation of hexosephosphates in the next stage was prevented. In the case of zymin, the liquid was filtered at this stage through starch-free paper and the insoluble residue washed free of reducing sugar with $N/10$ HCl, hydrolysed for 4 hours in the water-bath with 0·6 N HCl and the reducing power (expressed

as glucose) determined by Bertrand's method after neutralisation and treatment with "dialysed iron."

To the original filtrate from the zymin, or, in the absence of zymin, to the diluted and acidified fermentation fluid, were then added 2 vols. of absolute alcohol, in order to precipitate glycogen, yeast-gum or any other polysaccharide which might be present. This precipitate was removed by centrifuging and washed with 66·6 % alcohol to remove sugar, etc. It was then dried over H_2SO_4 and in a weighed portion the amount soluble in water was determined as well as the reducing power and rotation of this soluble portion before and after hydrolysis for three hours with 0·6 *N* HCl. All the rotations refer to the mercury green line.

In the filtrate from the precipitate produced by 66·6 % alcohol the phosphates and hexosephosphates were precipitated by adding baryta until the liquid was faintly alkaline to phenolphthalein, sufficient alcohol being added at the same time to keep the alcohol concentration constant at 66·6 volumes %. (All concentrations of alcohol are expressed as cc. of alcohol in 100 cc. of the mixture.) A 5 % solution of barium acetate in 66·6 % alcohol was then added until no further precipitate was produced and the precipitate filtered off, thoroughly washed with 66·6 % alcohol, finally washed with absolute alcohol and dried. This precipitate was in some cases used for the estimation of the relative amounts of mono- and di-phosphoric esters formed and hence, indirectly, of the total glucose esterified. (See later.)

The final filtrate from the barium salts was evaporated at 45°, 0·5 *M* Na_2SO_4 was added to remove the barium and it was then diluted to 100 cc. and filtered. The filtrate, which contained any unfermented sugar, was then examined for reducing power and rotation before and after hydrolysis with HCl.

The precipitate of barium salts was examined, when necessary, by decomposing a weighed portion of it with dilute sulphuric acid and in the filtrate determining

(*a*) the reducing power, by Bertrand's method;

(*b*) the free and total phosphorus (Neumann), and hence by difference the combined phosphorus.

The amounts of hexosemonophosphoric acid and hexosediphosphoric acid present, and from these their glucose equivalent were then calculated by the simultaneous equation shown below.

$$\text{g. of combined phosphorus} = \frac{X \times 31}{260} + \frac{Y \times 62}{340} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1).$$

$$\text{Direct reducing power (as g. of glucose)} = \frac{X \times 128}{260} + \frac{Y \times 72}{340} \quad \ldots\ldots\ldots\ldots\ldots (2).$$

X = g. of hexosemonophosphoric acid (Mol. wt. 260).

Y = g. of hexosediphosphoric acid (Mol. wt. 340).

This equation is based on the facts that the molecular reducing powers of the mono- and di-acids correspond respectively with those of 128 and 72 g. of glucose. The results are only approximate.

Experiments and Results.

Exps. 1 *and* 2. In a first experiment with zymin, in which fermentation was only continued for 2 hours, it was found that there was scarcely any production of a polysaccharide. A second similar experiment was therefore made in which the fermentation liquids were examined after 12 and 24 hours. The experimental details are given in Table I. The phosphate and water were added after incubation for 1 hour.

Table I. (*Exp.* 2.)

No. of flask	Weight of zymin (g.)	Volume of 10 % glucose solution (cc.)	Volume of toluene (cc.)	Volume of 0·6 *M* phosphate solution (cc.)	Volume of water (cc.)	Time of fermentation (hours)
1	2	15	0·2	—	—	1
2	2	15	0·2	2·5	—	After addition 12
3	2	15	0·2	—	2·5	,, 12
4	2	15	0·2	2·5	—	,, 24
5	2	15	0·2	—	2·5	,, 24

	Totals in 100 cc.				
	1	2	3	4	5
1. *Glucose converted into* CO_2, g.	0·043	0·821	0·397	0·948	0·594
2. *Residue.* Reducing power after hydrolysis (as g. glucose)	0·528	0·678	0·636	0·496	0·656
3. *Precipitate by* 66·6 % *alcohol*, g.	0·016	0·337	0·222	0·345	0·242
Rotation in 1 dm. tube	0	−0·13°	−0·30°	−0·13°	−0·34°
Reducing power, as g. glucose:					
(*a*) before hydrolysis	0	0	0	0	0
(*b*) after hydrolysis	0	0·023	0·014	0·019	0·012
4. *Baryta precipitate.* g. glucose combined as:					
(*a*) hexosemonophosphate	0·0002	0·008	0·0003	0·002	0·0004
(*b*) hexosediphosphate	0·036	0·156	0·062	0·115	0·069
5. *Final filtrate.* Reducing power, as g. glucose:					
(*a*) before hydrolysis	1·372	0·029	0·645	0	0·224
(*b*) after hydrolysis	1·396	0·132	0·713	0·116	0·297
Increase on hydrolysis	0·024	0·103	0·068	0·116	0·073

In this case there is evidence of production of a considerable proportion of polysaccharide, which is present in the final filtrate. The absolute quantity produced is greater in the two experiments in which phosphate was added (0·079 in 12 hours and 0·092 in 24 hours) than in the controls without added phosphate (0·044 in 12 hours and 0·049 in 24 hours), but it must be remembered that the amount of glucose converted into CO_2 was also greater in the former cases.

The ratios of the glucose converted into polysaccharide to the glucose fermented to CO_2 and to that converted into hexosephosphate were as follows:

	Experiment			
	2 12 hours Phosphate added	3 12 hours No phosphate added	4 24 hours Phosphate added	5 24 hours No phosphate added
$\frac{\text{Glucose to polysaccharide}}{\text{Glucose fermented to } CO_2}$	0·100	0·101	0·121	0·086
$\frac{\text{Glucose to polysaccharide}}{\text{Glucose converted into hexosephosphates}}$	0·609	1·68	1·14	1·43

It appears that the amount of glucose converted into this polysaccharide is roughly 10 % of the glucose fermented, but bears no definite relation to the amount of hexosephosphates produced. Only traces of a polysaccharide precipitated by 66·6 % alcohol from $N/10$ HCl solution were observed. It was, however, noticed that in Nos. 2, 3 and 5 the residue gave on hydrolysis a higher reducing power than the control. The cause of this was not ascertained.

Exp. 3. In order to obtain further information as to the nature of this polysaccharide an experiment on a somewhat larger scale was made. 50 g. of zymin, 37·5 g. glucose and 62·5 cc. of 0·6 M phosphate being used in a total volume of 437·5 cc. and incubated for 12 hours (Table II).

Table II. (*Exp.* 3.)

	Totals in 500 cc.	
	1	2
	Phosphate added	H_2O added
Final filtrate.		
Reducing power as g. glucose		
(*a*) before hydrolysis	1·271	14·629
(*b*) after hydrolysis	4·584	17·237
Increase on hydrolysis	3·313	2·608
Rotation in 1 dm. tube:		
(*a*) before hydrolysis	+1·16	+2·19
(*b*) after hydrolysis	+0·67	+1·73

As before, no glycogen was detected in the precipitate produced by 66·6 % alcohol, but yeast-gum was present and was detected by the formation of a bulky gelatinous precipitate with Fehling's solution.

The final filtrate, after removal of hexosephosphates and yeast-gum, contained a considerable amount of the polysaccharide as shown by the change in reduction (calculated on the whole amount) and rotation on hydrolysis with acid.

These two final filtrates were fractionated by precipitation with alcohol but no sharp separation of the polysaccharide was obtained. Thus in No. 1, in which the residual sugar (as measured by reducing power) was comparatively small (0·25 %), after the removal of a small amount of non-saccharine material (*A*) by 75 % alcohol, fractions containing polysaccharide were precipitated from the five times concentrated filtrate by 80 % (*B*) and by 95 % alcohol (*C*) and some of it was still present in the filtrate (*D*).

	A	*B*	*C*	*D*	*d*
Concentration of alcohol (% by volume)	75	80	95	—	95
Increase in reduction on hydrolysis (as g. of glucose)	0	0·412	0·082	0·526	0·494
Decrease in rotation in 1 dm. tube on hydrolysis (total volume 50 cc.)	0	0·445	0·04	0·67	0·72

In No. 2 (no phosphate added) the residual sugar concentration was much higher (4·15 %) and the results still less sharp. The purest product obtained

(*d*) closely resembled those obtained in the presence of added phosphate. This was precipitated from 24 cc. of a ten times concentrated solution by addition of 19 volumes of alcohol. It was then dissolved in 40 cc. of water, treated with 9 volumes of alcohol, filtered, evaporated to 8 cc. and precipitated by 19 volumes of alcohol. None of these precipitates gave a coloration with iodine or a precipitate with Fehling's solution.

Exps. 4 and 5. Experiments were next made in a similar manner with yeast-juice prepared by grinding top yeast (Table III).

Table III. (*Exp.* 4.)

No. of flask	Volume of yeast-juice (cc.)	Weight of glucose (g.)	Volume of toluene (cc.)	Volume of 0·6 *M* phosphate solution (cc.)	Volume of water (cc.)	Volume of 4 *N* HCl (cc.)	Time of fermentation (hours)
1	25	5	0·2	10	—	10	0·5
2	25	5	0·2	10	—	10	17·0
3	25	5	0·2	—	—	—	0
4	25	5	0·2	10	—	—	17·0
5	25	5	0·2	—	10	—	17·0

	Totals in 100 cc.		
	3	4	5
1. *Glucose converted into* CO_2, g.	0	1·354	0·537
2. *Precipitate by* 66·6 % *alcohol.*			
Reducing power as g. glucose:			
(*a*) before hydrolysis	0·011	0·009	0
(*b*) after hydrolysis	0·167	0·058	0·065
Increase on hydrolysis	0·156	0·049	0·065
Iodine reaction	+	−	−
3. *Baryta precipitate.*			
g. glucose combined as:			
(*a*) hexosemonophosphate	0·083	0·305	0·061
(*b*) hexosediphosphate	0·100	0·412	0·122
4. *Final filtrate.*			
Reducing power as g. glucose:			
(*a*) before hydrolysis	4·628	2·224	3·777
(*b*) after hydrolysis	4·831	2·712	4·249
Increase on hydrolysis	0·203	0·488	0·472
Rotation in 1 dm. tube:			
(*a*) before hydrolysis	+3·00°	+2·09°	+3·05°
(*b*) after hydrolysis	+2·69°	+1·43°	+2·49°
5. Total glucose accounted for, g.	5·181	4·841	5·034
Glucose not accounted for, g.	—	0·34	0·147

An attempt was made in this case to account for the whole of the carbohydrate, both that originally present in the yeast-juice and that added as glucose. Actually, discrepancies of 6·6 % and 2·9 % were found. In order to obtain a more accurate estimate of the CO_2 produced by fermentation, the combined CO_2 present in the liquid before and after fermentation was estimated in parallel samples by adding excess of hydrochloric acid (saturated with CO_2), measuring the volumes evolved, and correcting for any difference in the values obtained before and after fermentation.

The original juice contained a certain amount of glycogen, for the precipitate produced in the acid solution by 2 volumes of alcohol gave the iodine reaction for this substance. This disappeared during fermentation and no glycogen was detected in either of the fermented samples. It also contained a considerable amount of polysaccharide soluble in 66·6 % alcohol, as the final filtrate gave an increase of reducing power (equivalent to 0·203 g. glucose) and a decrease of rotation (0·31° in 1 dm. tube) on hydrolysis.

The total polysaccharides of the original juice (expressed as g. of glucose) and of the two fermented juices were as follows:

	3	4	5
Precipitated by 66·6 % alcohol	0·158	0·048	0·065
In final filtrate	0·203	0·488	0·472
	0·361	0·536	0·537

There was, therefore, an increase on fermentation corresponding at least to 0·167 g. glucose and this increase was in this case the same in the presence and absence of added phosphate, although the amount of CO_2 evolved in the absence of added phosphate (0·54 g.) was much less than in its presence (1·354 g.).

Table IV. (*Exp.* 5.)

No. of flask	Volume of yeast-juice (cc.)	Weight of glucose (g.)	Volume of toluene (cc.)	Volume of 0·6 *M* phosphate solution (cc.)	Volume of water (cc.)	Duration of fermentation (hours)
1	230	46	2	92	0	17
2	230	46	2	0	92	17

(No. 1) I. 230 cc. of final filtrate + 4 volumes of absolute alcohol.
Precipitate *A*; dissolved in 100 cc. of water.

II. Filtrate from I evaporated down to 100 cc. at 45° + 9 volumes of alcohol.

III Precipitate from II dissolved in 50 cc. of water + 9 volumes of absolute alcohol.

IV. Precipitate from III dissolved in 20 cc. of water + 3 volumes of absolute alcohol.
Precipitate *B*; dissolved in 50 cc. of water.

V. Filtrate from IV + 1·5 volumes of absolute alcohol.
Precipitate *C*; dissolved in 50 cc. of water.

The final filtrate of No. 2 was treated in a similar manner and yielded corresponding fractions, *a*, *b* and *c*.

	No. 1			No. 2		
	A in 100 cc.	*B* in 50 cc.	*C* in 50 cc.	*a* in 100 cc.	*b* in 50 cc.	*c* in 50 cc.
Reducing power (as glucose):						
(*a*) before hydrolysis	0·144	1·075	0·304	0·096	0·626	0·409
(*b*) after hydrolysis	0·371	1·973	0·562	0·328	1·359	0·702
Increase on hydrolysis	0·227	0·898	0·258	0·232	0·733	0·293
Rotation in 1 dm. tube:						
(*a*) before hydrolysis	+0·66°	+4·23	+1·11	+0·33	+3·02	+1·32
(*b*) after hydrolysis	+0·22	+2·35	+0·64	+0·19	+1·60	+0·74

Parallel with the foregoing a larger scale experiment (Exp. 5) was carried out with two quantities of 230 cc. of the same preparation of yeast-juice + 45 g. of glucose, to one of which (1) 92 cc. of 0·6 *M* phosphate were added and to the

other (2) 92 cc. H_2O. The final filtrates were then fractionated with alcohol and the resulting precipitates (see Table IV) examined. As before, a considerable amount of polysaccharide was found in each of these. As *B* and *b* contained the largest quantity these were submitted to refractionation by 80 and 90 % alcohol and thus divided into three fractions, which were then examined (Table V).

Table V. (*Exp.* 5.)

	Totals in 50 cc.					
	X Precipitated by 80 %	*Y* Precipitated by 90 %	*Z* Soluble in 90 %	*x* Precipitated by 80 %	*y* Precipitated by 90 %	*z* Soluble in 90 %
Reducing power as g. glucose:						
(*a*) before hydrolysis	0·278	0·117	0·580	0·165	0·094	0·476
(*b*) after hydrolysis	0·762	0·262	0·803	0·462	0·187	0·650
Increase on hydrolysis	0·484	0·145	0·223	0·297	0·093	0·174
Rotation in 1 dm. tube:						
(*a*) before hydrolysis	+2·05°	+0·67°	+1·47°	+1·29°	+0·47°	1·18°
(*b*) after hydrolysis	+0·92°	+0·29°	+0·90°	+0·45°	+0·15°	0·60°

The purest of these fractions was probably *X*, and, if it can be assumed that it was free from glucose, its rotatory and reducing powers, expressed in terms of those of the glucose formed from it by hydrolysis, are rotation 2·16, and reduction 0·36; *x* possessed almost the same characteristics; rotation 2·26, reduction 0·36.

Exp. 6. Another large-scale experiment with 500 cc. of yeast-juice gave very similar results (Table VI). The final filtrate from the original yeast-juice was found to contain a considerable amount of the polysaccharide, which had precisely similar properties to that isolated after fermentation and behaved in the same manner on fractionation. This amount diminished when the yeast-juice was incubated for 17 hours in the absence of added sugar, but increased in the sample to which sugar and phosphate were added before incubation.

In this case the original yeast-juice and the liquid after fermentation with glucose and phosphate for 17 hours contained practically the same amount of glycogen, whilst the sample which was incubated without sugar for 17 hours had lost its glycogen. Whether this indicated merely a delayed hydrolysis of the original glycogen in presence of glucose and phosphate or an actual new formation could not be decided.

Exp. 7. Finally, to complete the series, an experiment was made with maceration extract prepared by Lebedev's method from a dried mild ale top yeast (Table VII). This contained very little polysaccharide either in the precipitate produced by 66·6 % alcohol or in the final filtrate and also contained practically no hexosephosphate. Very little polysaccharide was found in the final filtrate after incubation with glucose alone or in presence of excess of phosphate (10 cc. of 0·6 *M* to 25 cc. juice), although a moderately vigorous

evolution of CO_2 occurred. In experiments in which only 5 cc. of the phosphate solution were added and in which a much more vigorous fermentation occurred, there was a decided formation of the polysaccharide, both from glucose and fructose. The products were not further investigated.

No glycogen was produced in this experiment.

Table VI. (*Exp.* 6.)

No. of flask	Yeast juice (cc.)	Glucose (g.)	Toluene (cc.)	0·6 *M* phosphate (cc.)	Duration of fermentation (hours)
1	500	0	0	0	0
2	500	50	5	200	17
3	100	0	1	0	17

Calculated for 100 cc. yeast juice.

Final filtrate.	1	2	3
Reducing power as g. glucose:			
(*a*) before hydrolysis	0·017	0·618	0·041
(*b*) after hydrolysis	0·619	1·401	0·605
Increase on hydrolysis	0·602	0·783	0·564

Fractionation by alcohol of final filtrates 1 and 2. $\frac{4}{10}$ of each filtrate (after removal of alcohol) was used for this purpose.

1. 80 % precipitate. 400 cc. evaporated to 200 cc. and 4 volumes of alcohol added. The precipitate was dissolved in 60 cc. of water and precipitated by 4 volumes of alcohol, this process being repeated twice.
2. 85 % precipitate. The filtrate from 1 was evaporated to 80 cc. and made up to 85 % with alcohol. The precipitate was then dissolved up and reprecipitated with 85 % alcohol and this process repeated twice.
3. 90 % precipitate. The filtrate from 2 was made up to 90 % by the addition of alcohol and the precipitate redissolved in 30 cc. and reprecipitated by 90 % alcohol.
4. Residue. The filtrate from 3 was evaporated to 100 cc.

Examination of the fractions obtained from final filtrates 1 and 2.

(Each purified fraction was made up to 100 cc. with water.)

Rotation (1 dm.)			1	2
80 % precipitate	(*a*) before hydrolysis		+0·56°	+1·65°
	(*b*) after	,,	+0·20	+0·52
85 % ,,	(*a*) before	,,	+0·68	+0·74
	(*b*) after	,,	+0·22	+0·26
90 % ,,	(*a*) before	,,	+0·09	+0·12
	(*b*) after	,,	+0·02	+0·04
Residue	(*a*) before	,,	−0·50	−0·63
	(*b*) after	,,	−0·26	−0·30
Reducing power (as g. glucose)				
80 % precipitate	(*a*) before hydrolysis		0·010	0·086
	(*b*) after	,,	0·348	0·917
85 % ,,	(*a*) before	,,	0·083	0·070
	(*b*) after	,,	0·423	0·423
90 % ,,	(*a*) before	,,	0	0
	(*b*) after	,,	0·054	0·073
Residue	(*a*) before	,,	0·061	1·646
	(*b*) after	,,	0·115	1·885

Conclusions.

The foregoing experiments afford abundant confirmation of the observation made by Harden and Young of the enzymic production of polysaccharides in yeast-juice and similar preparations. Substances of a similar character are present in freshly prepared yeast-juice in addition to glycogen and yeast-gum.

Table VII. *Exp.* 7. 1. xii. 1925.

(Maceration juice from dried mild ale yeast. 14. iv. 1925)
(With 0·2 cc. toluene in each experiment)

Date	Exp. no.	Maceration juice (cc.)	Glucose (g.)	0·6 *M* phosphate mixture (cc.)	Water (cc.)	Duration of fermentation (hours)
1. xii. 1925	1	25	0	0	0	0
,,	2	25	5	0	0	0
,,	3	25	0	0	0	17
,,	4	25	5	0	10	17
,,	5	25	5	10	0	17
3. xii. 1925	6	25	5	5	0	17
5. xii. 1925	7	25	2·5	5	0	18
,,	8	25	2·5*	5	0	18

* Fructose (B.D.H.).

		1	2	3	4	5	6	7	8
1	*Glucose converted into* CO_2	0	0	0	0·677	0·464	—	—	1·276
2	*Precipitated by* 66·6 % *alcohol.*								
	Reducing power as g. glucose:								
	(*a*) before hydrolysis	0·004	0·015	0·003	0·027	0·004	0·020	0·010	0·007
	(*b*) after hydrolysis	0·054	0·135	0·095	0·203	0·106	0·195	0·120	0·109
	Iodine reaction for glycogen	–	–	–	–	–	–	–	–
3.	*Baryta precipitate.* g. glucose combined as:								
	(*a*) hexosemonophosphate	0	0	0	0·667	0·368	0·034	0·174	0·258
	(*b*) hexosediphosphate	0	0	0	0·002	0·119	1·083	0·753	0·180
4.	*Final filtrate.*								
	Reducing power as g. glucose:								
	(*a*) before hydrolysis	0	4·859	0	3·243	3·712	2·041	0·008	0·002
	(*b*) after hydrolysis	0	4·838	0	3·238	3·735	2·174	0·130	0·179
	Increase on hydrolysis	0	−0·021	0	−0·005	0·023	0·133	0·122	0·177
	Rotation in 1 dm. tube (total volume 100 cc.):								
	(*a*) before hydrolysis	−0·17°	+2·75°	−0·23°	+1·75°	+2·30°	+1·34°	+0·05°	+0·575°
	(*b*) after hydrolysis	−0·14°	+2·80°	−0·18°	+1·78°	+2·20°	+1·15°	−0·04°	+0·140°

The formation of these substances was found to be irregular and no definite conditions were found which specially favoured it. In particular no relation was found between the amount of phosphoric esters formed and the degree of polysaccharide formation. No definite evidence of the production of glycogen was obtained in these experiments.

Among the compounds formed are substances which are probably dextrins, possessing about twice the rotatory power and about 36 % of the reducing power of the glucose formed from them by hydrolysis[1].

The investigation is being continued.

I desire to express my thanks to Prof. A. Harden for the valuable advice and assistance he has given me in the prosecution of this investigation.

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[1] All the products obtained contained a certain amount of mineral matter.

CVIII. A METHOD FOR DETERMINING THE ACCURACY OF ANALYSES OF ETHYL IODIDE VAPOUR FOR MEASUREMENTS OF THE CIRCULATION OF THE BLOOD IN MAN.

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The use of ethyl iodide, inhaled as a very dilute vapour in air, for measuring the circulation of the blood in man seems now to be assured of extensive adoption [see Mobitz, 1926]. For this measurement samples of inspired, expired, and alveolar air of about 250 cc. each, containing from 0·2 to 0·5 mg. of ethyl iodide, are analysed by passing them through a U-tube of iodine pentoxide heated to about 170°. The iodine thus liberated is collected by passing the effluent air through 10 cc. of 5 % potassium iodide solution. It is then measured by titrating the solution with sodium thiosulphate with starch as the indicator in the usual manner.

This method of analysis is well known for determining carbon monoxide, and has a wide applicability to volatile organic substances. It is essential, however, to determine the reliability of the particular preparation of iodine pentoxide employed, and also to determine the speed, or volume per minute, at which air containing the vapour can be passed without allowing any appreciable amount to escape oxidation. It has been found that the rate of flow (about a litre a minute) advised in the paper of Henderson and Haggard [1925] describing the ethyl iodide method is initially much too rapid for complete oxidation. This does not invalidate the circulation rates determined by analyses so conducted, for the reason that it is the relative, not the absolute, amounts of iodine and ethyl iodide found in the three airs from which the circulation of the blood is calculated. If all three analyses are too low by x % the circulation is as correctly estimated as if the analyses were precise.

In the desire for a more nearly absolute precision, however, the following method of checking the accuracy of analysis has been worked out. It may be mentioned here incidentally that when the glass wool, in which the iodine pentoxide is held, turns brown the colour is probably due to iron, not iodine, and does not affect the analyses.

Make a micropipette as follows: warm a thermometer which has a narrow capillary and reads up to 250° or 300° until the greater part of the capillary is full of mercury. Break off the bulb and the top of the thermometer. Note

the number of degrees of the graduation through which the column of mercury extends. Blow the mercury out into a tared watch-glass and weigh. From this weight, the specific gravity of mercury and the number of degrees (noted above), calculate the volume in per cent. of a cubic millimetre represented by one degree on the thermometer. Multiply by 1·944 (the specific gravity of ethyl iodide), and thus obtain the weight of ethyl iodide corresponding to one degree on the thermometer. If the capillary is as small as it should be, the volumes used are from 25° to 50°, which give 0·5 to 1·0 mm.^{3}, and weights of 1 to 2 mg. of ethyl iodide.

Fit a two-holed rubber stopper into the neck of a 200 cc. bottle. The micropipette is inserted up to its middle, or a little more, through one hole. A glass tube passes through the other hole and down to the bottom of the bottle. (See Fig. 1.)

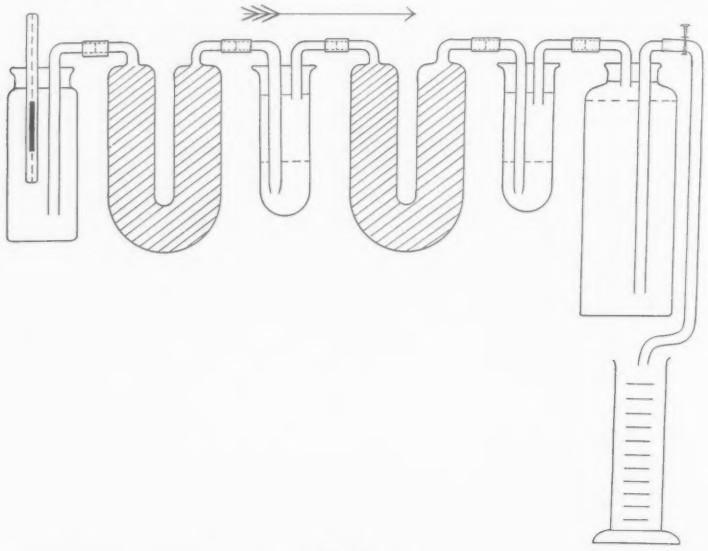

Fig. 1.

The stopper is taken out and held so that the micropipette is horizontal. A drop of ethyl iodide is touched to the end of the capillary and a column 60° or 70° in length is allowed to run along the capillary. The excess is then wiped off the end, and the length of the column is reduced by touching the end of the capillary with a finger. Then the pipette is tilted until the column runs a few millimetres away from the end. Any of the ethyl iodide on the surface of the pipette is evaporated off by fanning.

The bottle is slipped on over the pipette and glass tube and the stopper is pressed in. Then the bottle is stood upright. The glass tube through the stopper is connected by a rubber tube to the U-tube of iodine pentoxide which is to

be tested. From the other side of this U-tube a glass tube passes down through a two-holed rubber stopper into a large, thick-walled, test-tube containing 10 cc. of 5 % potassium iodide solution. A second glass tube through the other hole in this stopper enables this test-tube to be connected to a second U-tube of iodine pentoxide which in turn leads to a second test-tube containing potassium iodide solution. The two U-tubes stand side by side in an air-bath heated electrically to 170°. From the second test-tube a rubber tube leads to a bottle containing 4 or 5 litres of water. The water in this bottle is drained off through a vertical tube, and a current of air is thus drawn through the whole train.

After air has thus been drawn through the micropipette for 10 or 15 seconds, thus sweeping the ethyl iodide into the bottle, the stopper in the 200 cc. bottle is loosened enough to allow air to pass in around the stopper.

The flow of air through the train is measured by means of a graduated cylinder into which the large bottle of water is drained. The flow should be set at 300 cc. a minute for 2 minutes, then at about a litre per minute for 4 or 5 minutes. The first period draws the ethyl iodide into the U-tube, the second, at the more rapid rate, washes the iodine liberated into the potassium iodide solution. No iodine escapes at even the most rapid flow in the second period. But it is possible that when the flow in the first period is too rapid some ethyl iodide may escape oxidation in the first U-tube, and in part also even in the second. If this occurs, the experiment should be repeated with an air flow of 200 cc. or 100 or even 50 cc. per minute until 500 or 600 cc. have been drawn through the train. Then a rapid flow should be maintained for 4 or 5 minutes to wash out the iodine. That iodine, once liberated, is not lost by a rapid flow is easily demonstrated by putting a second test-tube containing a little starch paste into the train just after the first test-tube. That ethyl iodide may escape complete oxidation in the first U-tube is shown by the appearance of iodine in the test-tube placed after the second U-tube.

That the rate of flow is correct, and that the U-tube of iodine pentoxide is working properly, is demonstrated by finding that the fluid in the first test-tube requires on titration an amount of thiosulphate corresponding to the amount of ethyl iodide put into the micropipette.

For the standardisation of the titration weigh out exactly 50 mg. of iodine and dissolve in 100 cc. of 5 % solution of potassium iodide. Weigh out approximately 1·1 g. of sodium thiosulphate and dissolve in 1000 cc. of distilled water. Using starch paste as the indicator titrate 5 cc. of the iodine solution and calculate the amount of iodine in mg. decolorised by 1 cc. of thiosulphate. This amount multiplied by 0·341 gives the amount of ethyl iodide which on oxidation with iodine pentoxide should liberate enough iodine to require 1 cc. of thiosulphate. The factor 0·341 is derived from the figure 0·81, which is the weight of iodine in 1·0 of ethyl iodide, and from the equation of oxidation

$$10\ C_2H_5I + 13\ I_2O_5 = 20\ CO_2 + 25\ H_2O + 36\ I.$$

In recent tests of U-tubes of iodine pentoxide by this procedure, I have

obtained results balancing to a difference of less than 1 %. A balance to within 5 % is, however, usually sufficient owing to the fact that the calculation of the volume of the circulation of the blood through the lungs depends, not upon the absolute, but upon the relative amounts of ethyl iodide found in the inspired, expired and alveolar airs.

In conclusion I wish to express my thanks to Mr Francis H. Carr of the British Drug Houses for very practical assistance in developing satisfactory U-tubes of iodine pentoxide without which the general introduction of the method in England would be greatly impeded. Especially am I indebted to Prof. Joseph Barcroft for the hospitality of his laboratory, and to Mr Henry Barcroft for collaboration.

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CIX. FEARON'S "PYROGALLOL" TEST AS A POSSIBLE BASIS FOR THE ESTIMATION OF VITAMIN A.

By STANLEY GORDON WILLIMOTT and THOMAS MOORE.

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(*Received July 7th, 1926.*)

With a view to finding a satisfactory basis for the quantitative trial of a colour test for vitamin A against parallel feeding experiments we have examined both the arsenic trichloride test [Rosenheim and Drummond, 1925] and Fearon's pyrogallol test [1925]. Having regard especially to the fact that in these tests the rôles of arsenic trichloride and trichloroacetic acid (not specially purified [Carr and Price, 1926]) are interchangeable we have come to the opinion that the two tests are not altogether independent. Since, *ceteris paribus*, the pyrogallol test is to be preferred to the arsenic trichloride test both on account of the permanence of its colour and of the safety of its reagents we have made it the subject of a detailed survey.

The Fearon test.

As described by its author this test consists in the treatment of a few drops of the given oil with crystals of pyrogallol and 5 cc. of a 12 % solution of trichloroacetic acid in light petroleum. A permanent pink colour is produced on boiling, on standing or on the addition of benzoyl peroxide, the depth of which was suggested as the basis of a colorimetric estimation.

Seeking to improve the test we employed wide ranges of phenols and condensing agents, but in the end only altered the phenol, finding resorcinol to produce a better pink than did pyrogallol. However, on applying the test, with this one modification, upon varying amounts of cod-liver oil results were obtained erratic both in depth and in shade. A further study of the test was necessary.

The effect of moisture.

Fearon has insisted that the solvent employed, whatever it be, must be rigorously dried. Our first care, therefore, was to examine the effect upon the colour of traces of water. When the latter was added in small quantity after the formation of the colour we found, instead of decoloration, a clear separation of the colour into the aqueous layer[1]. Traces of water, therefore, did not seem objectionable except if causing cloudiness. But could such water prevent the formation of colour?

[1] This phenomenon was observed when the test was carried out as described by Fearon, using light petroleum as a solvent. Under other conditions decoloration did not always take place.

To answer this question a little oil was mixed with the trichloroacetic acid solution without the addition of resorcinol, the mixture at once being extracted with three drops of water. On testing with resorcinol the aqueous layer gave a deep carmine, the light petroleum layer only a faint pink. Water, therefore, does not prevent the formation of colour when sufficient acid is present. Its use, in fact, might provide a possible method for the isolation of the "vitamin" condensation products.

Both toluene and light petroleum, without special drying, proved efficient solvents, but owing to its lower volatility we adopted the former.

Oxidation and the effects of heating.

On standing in air, even after boiling, the colour developed both slowly and irregularly. Consequently benzoyl peroxide was used to hasten its formation. Too high concentrations of the latter, or heating even at low concentrations, caused a fading of the colour to brown, but by using 0·5 cc. of a saturated toluene solution of the peroxide in every test a regular colour development occurred in a reasonable time without any fading in the first few hours.

The stages of colour development deserve mention. On mixing cod-liver oil with the trichloroacetic acid solution there is a slight deepening in tint. On prolonged boiling a further deepening to sage green occurs, and a pink is no longer given with resorcinol. On the other hand, if resorcinol is added immediately on mixing the oil with the acid a faint pea-green usually results, which changes readily into the normal shade of pink.

Variations with resorcinol concentration.

In Fearon's technique the colouring agent, being almost insoluble in light petroleum, is added in the form of crystals. This, of course, ensures its ultimate supply in adequate quantity, but in solutions left to stand overnight we often noticed that such crystals had become more coloured than the bulk of the solution, pointing to imperfect colour development. As an improvement, therefore, we added our resorcinol in ethereal solution, and by so doing were able to make a quantitative study of the effect of this factor.

Table I.

Colours formed by eight drops of cod-liver oil treated with 5 cc. of 10 % trichloroacetic acid in toluene, 0·5 cc. of saturated benzoyl peroxide in toluene and one drop of ethereal resorcinol at each of the concentrations shown.

	Resorcinol concentration					
Time	0·25 %	0·5 %	1 %	2 %	4 %	8 %
Before adding peroxide	Faint pink	Faint pink	Faint green	Faint green	Faint green	Faint green
20 minutes later	Deep purple	Deep purple	Pink	Pink	Pale pink	Pale yellowish pink
2 hours later	Fading purple	Fading purple	Deep purple	Deep reddish purple	Carmine	Carmine

These results are of importance, especially since the colour change from purple to carmine was not merely one of depth, but a sharp transition from blue to red. Moreover, although without resorcinol no colour other than brown was obtained, the colour produced with but little resorcinol present was both denser and more rapid in formation than at higher concentrations. An adjustment of resorcinol concentration, therefore, was obviously essential to the proper working of the test.

The point at which the break occurred may be calculated at about 0·5 % of resorcinol on the amount of oil taken, and with pyrogallol a very similar phenomenon was observed with a break at about the same point. As an explanation of the formation of two distinct colours we might suggest, purely as an hypothesis, that while below 0·5 % two "vitamin" molecules combine per resorcinol molecule, above 0·5 % only one such molecule combines.

Quantitative technique.

Since the rapidity of colour formation varies in an inverse manner with the resorcinol concentration, it was necessary to employ such a concentration of the latter as to cause a full carmine coloration in a reasonable time without the formation of purple. The test was therefore carried out with resorcinol present to the extent of 0·5 % of the oil taken. The oil was first dropped in varying quantities into dry test-tubes. The appropriate quantity of ethereal resorcinol was added, and then, with thorough vertical shaking, 5 cc. of 10 % trichloroacetic acid in toluene and 0·5 cc. of benzoyl peroxide in the same solvent. After a period of 2 hours the colours were compared against a solution of magenta toned with methylene blue. Results are appended.

Table II.

	Drops of cod-liver oil			
	2	4	6	8
	Colour densities			
1st Exp.				
(T. M.)	5·6	9·8	15·2	19·0
2nd Exp.				
(T. M.)	5·6	9·8	15·7	21·7
(S. G. W.)	5·2	10·4	14·3	19·8
3rd Exp.				
(T. M.)	4·8	10·0	14·7	19·6
(S. G. W.)	4·5	9·4	14·2	18·4
Average of 2nd and 3rd Exps.	5·0	9·9	14·7	19·8

From these figures it will be seen that the colour developed was fairly accurately proportional to the amount of oil taken. However, in order to exclude the obvious criticism that the colour depths were determined not by the oil but by the resorcinol added, another experiment was carried out keeping the latter constant at one drop of 8 % solution in every case.

Table III.

Time (hours)	Drops of cod-liver oil: 2	4	6	8
	Colour densities			
2	4·7	8·1	12·7	16·0
3	5·7	9·8	14·8	19·7
4	—	—	—	22·1
5	6·0	11·4	17·0	22·7
8	—	—	—	23·5 (brownish)

The linear agreement still holds good, but the colours are slow in reaching their maximum values. The best method of overcoming this difficulty is still to be decided, but in the meantime it is proposed to fix a definite time and temperature for colour development.

The specificity of the test.

In this work our primary purpose has been to place the test upon a quantitative basis, but from a qualitative standpoint we have also confirmed much of Fearon's evidence as to its specificity. Positive results were obtained with all cod-liver oils, with beef-suet, with butter-fat and with egg-yolk-fat; negative with olive oil, linseed oil, palm kernel oil and "ostelin" (a preparation rich in vitamin D). A sample of Newfoundland cod-liver oil was found to give a coloration about forty times as dense as that given by a sample of butter-fat.

SUMMARY.

(1) Fearon's test for "vitamin A," suitably modified, is shown to be capable of quantitative application.

(2) Unless the concentration of resorcinol (or pyrogallol) is suitably controlled a deep purple may develop in place of the usual carmine.

(3) Under certain conditions it is possible to extract the "vitamin" condensation products with a little water.

We desire to express our cordial thanks to Sir F. G. Hopkins for his support during the course of this work. We are also indebted to Messrs Parke, Davis and Co., to Mr Norman Evers of Messrs Allen and Hanbury, and to Messrs Southall Bros. and Barclay for their assistance in supplying information and for samples of oil.

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CX. STUDIES ON THE EFFECT OF HEAT ON MILK.

I. SOME PHYSICO-CHEMICAL CHANGES INDUCED IN MILK BY HEAT.

By HUGH EDWARD MAGEE and DOUGLAS HARVEY.

From the Rowett Research Institute, Aberdeen.

(*Received May 26th, 1926.*)

Experience has proved that pasteurisation of low-grade cow's milk makes it safer for human consumption than similar milk in the raw state. The process also helps to preserve its value as a marketable commodity for many hours after being milked; but the question whether its nutritive properties are detrimentally affected cannot yet be taken as settled. Opinions on the matter are very conflicting and further investigation appeared therefore desirable. The problem has engaged the attention of several workers at this Institute for the past $2\frac{1}{2}$ years.

Perhaps no one constituent of milk is of more importance than another for the building up of new tissue in the young animal. There is, however, evidence that the calcium and phosphorus contents occupy an important position in this respect. Thus, Telfer and Crichton [1924] showed that comparatively trifling reductions in the calcium and phosphorus of goat's milk had profound effects on the young goats fed on it. It may be also, as suggested by Bunge [1904] and more recently by Hunt, Winter, *et al.* [1923], that the physical state of the calcium and phosphorus of the diet is of some importance for their proper assimilation by young and adult animals alike.

Since Sydney Ringer [1890] showed that addition of soluble calcium salts to heated milk restored its coagulability by rennin it has been generally accepted that, of all the constituents of milk, calcium was the one most affected by heat. It follows from the work of Van Slyke and Bosworth [1914], György [1923] and others, that calcium is found in two forms in milk, some in soluble or in ionic form and some organically bound with caseinogen in the form of a protein salt. In comparatively recent times several estimations of the relative amounts of soluble and colloidal calcium in fresh milk have been made, but attempts to determine whether the ratio is affected by heat have been very few.

Grosser [1913] found a loss of 5·4 % of soluble CaO in cow's milk (expressed as percentage of the total CaO) after boiling 15 minutes. The amounts of

soluble P_2O_5 and N were only slightly affected. In human milk, however, after boiling 15 minutes the losses were, CaO 21%, P_2O_5 7 % and N 12 % of the respective totals. Bell [1925] found that when cow's milk was kept at 77° for 30 minutes the soluble CaO fell by 4·6 % of the total. His results cannot, however, be accepted as confirmation of Grosser's because the degree and duration of heat were not the same in the two cases.

The experiments recorded in this paper were directed to determine what alterations were brought about in the state of some of the milk constituents by varying amounts of heat. Attention was focused chiefly on the calcium content for reasons which will be obvious from the foregoing. Two other papers in this series will appear later dealing with the relative effects of feeding fresh and heated milk on (*a*) the metabolism of young animals as determined in balance experiments, and on (*b*) the rate of growth of groups of young animals.

Experimental.

The research was directed broadly along three lines: the effect of heat on (*a*) the calcium content, (*b*) the colloids, and (*c*) the digestibility of milk.

Determination of soluble calcium. This was determined (1) by precipitation of the proteins with colloidal ferric hydroxide; (2) in rennet whey, and (3) by dialysis of milk for varying lengths of time.

Exp. 1. *Precipitation of proteins with colloidal ferric hydroxide.* According to Rona and Michaelis [1909] colloidal ferric hydroxide splits up the "casein-calcium compound" of milk and 70–80 % of the total CaO appears in soluble form, most of the P_2O_5 being carried down as an adsorbate. Kay [1925] has confirmed the disappearance of soluble P_2O_5 by this method of precipitation. Very probably this action of colloidal ferric hydroxide is due to the free Cl′ its solutions contain [Pauli, 1922].

In four samples of whole fresh milk we found an average of 85·1 % of the total CaO in soluble form and 0·0122 % of P_2O_5. In four samples of whole milk pasteurised at 65° for 30 minutes, we found 71·3 % of CaO and 0·0117 % of P_2O_5 in soluble form. These results confirm those of Rona and Michaelis and indicate that about 14 % of the total CaO was changed by heat into a form that the colloidal ferric hydroxide could not attack.

Exp. 2. *Coagulation by rennin.* The three kinds of milk used for all the experiments which follow are defined as under:

(1) Fresh milk: skimmed cow's milk 3 hours after milking.

(2) Pasteurised milk: (1) kept at a temperature of 70° for 30 minutes in a flask immersed in a water-bath.

(3) Boiled milk: (1) kept in a flask immersed in a water-bath at boiling point for an hour.

Reflux condensers were fitted to the flasks to prevent loss of water during the heating processes. The baths of water were quickly brought to the desired temperature and the flasks of milk were kept in the baths while the temperature

was being raised. After heating, the milk was cooled rapidly. There was never any skin formed on the surface of the milk.

The p_H of milk is not altered by rennin coagulation [Milroy, 1915; György, 1923]. Most probably, therefore, splitting up of the colloidal calcium does not occur and all the soluble calcium is present in the whey except what may be carried down by adsorption. Heated milk is very slowly acted on by rennin or not at all. To make it coagulable $CaCl_2$ in solution was added. No buffer mixture was added to counteract the slight lowering of the p_H caused by the addition of $CaCl_2$ [Milroy, 1915]. The commercial rennin used, originally of p_H 2·8, was dialysed for 24 hours in running tap water and the p_H became 6·1, the same as that of the tap water. The rennin was still quite active and contained only an insignificant amount of CaO, 0·008 %.

5 cc. of this solution and 5 cc. of a solution of $CaCl_2$ containing 0·0252 g. CaO were added to 50 cc. each of fresh, pasteurised and boiled milk in 100 cc. centrifuge tubes, mixed, kept at 40° until coagulated and centrifuged for 20 minutes. The $CaCl_2$ added was the minimal amount necessary to start coagulation in the boiled milk. 40 cc. of whey were pipetted off and ashed, the calcium precipitated as oxalate and titrated with $N/10$ $KMnO_4$. From the data obtained, the amount of soluble CaO in 100 cc. of milk was calculated, assuming that the concentration was the same in the coagulum as in the whey. (The amount of CaO in the $CaCl_2$ added was, of course, deducted.) For reasons given above, the assumption is probably not quite correct, but we were not aware of any means of correcting for the loss due to adsorption. All experiments were done in duplicate.

Table I.

No.	Total CaO %	cc. $N/10$ $KMnO_4$ for 40 cc. whey		
		Fresh	Pasteurised	Boiled
1	0·1610	11·00	10·00	9·80
2	0·1663	10·30	10·10	9·30
3	0·1743	9·60	9·10	9·15
4	0·1739	11·80	11·37	11·15
5	0·1834	10·69	10·10	8·76
6	0·1736	10·00	9·67	8·99
7	0·1729	9·62	9·47	9·70
8	0·1757	12·36	12·30	11·65
Av.	0·1726	10·67	10·26	9·81
CaO as % of total		22·72	20·73	18·52

The results (Table I) show on the average a loss in pasteurised milk of 2 %, and in boiled milk of 4 %, of soluble CaO expressed as percentage of the total CaO.

Exp. 3. *Dialysis of milk.* By dialysing milk against running water all the soluble calcium will in time pass out and the amount so lost can be estimated by difference. The CO_2 dissolved in the water, however, complicates the method. Being diffusible it can break up the Ca caseinate, precipitating the protein and bringing the Ca into solution as $Ca(HCO_3)_2$. Most probably, however, this

action would not occur to any appreciable degree until the buffer action of the soluble phosphates had ceased on their complete disappearance from the inner fluid. Prior to this the observed rate of loss of soluble calcium should give a fairly accurate picture of the relative concentration of soluble calcium in different kinds of milk since the rate of loss is directly proportional to the concentration.

Fat extraction thimbles 4·3 × 12·3 cm. were impregnated with collodion meth. (B.P.) as described by Hatschek [1920]. After practice membranes of approximately equal permeability could be prepared. Care was taken that the sacs were of like permeability by testing them on the same sample of milk for a certain time. 20 cc. of fresh, pasteurised and boiled milk were pipetted into each of three sacs suspended in running tap water in a wide-mouthed vessel. The depth of the water was such that, when, owing to the osmotic pressure of the milk, the volume inside the sacs was greatest, the sacs remained floating. Thus, approximately equal areas of membrane surface were in contact with water. Nine sacs at a time—three for each kind of milk—were accommodated in the vessel and care was taken that good circulation of water was maintained round each sac. After dialysis the amount of CaO in the contents of each sac was determined. The addition of a preservative to the milk was considered unnecessary because, as the experiments were carried out in winter, the temperature of the water was never more than a few degrees above 0°. It is to be remembered also that lactose would soon diffuse out. The formation of lactic acid was therefore considered highly improbable.

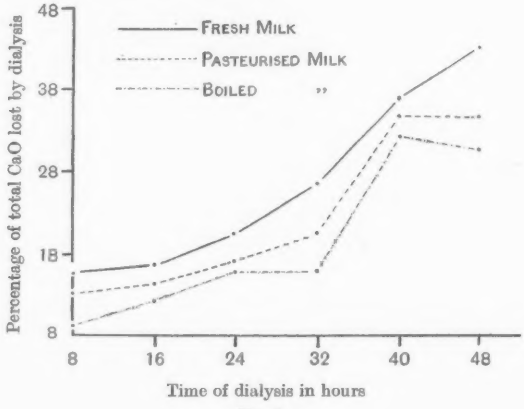

Fig. 1.

Dialysis was carried out for 8, 16, 24, 32, 40 and 48 hours. The results expressed as loss per cent. of the total CaO are shown graphically in Fig. 1. Each point is the average of nine analyses of dialysates, except at 8 and 24

hours where each is an average of three. Several other experiments were done at these times using sacs made from 4 % acetic collodion which were found more permeable than those made with "collodion meth." The former gave results showing similar relative differences as between the three kinds of milk, and therefore acted as a check on the results obtained with the "collodion meth." sacs; but the losses were too great to warrant their inclusion with the latter for averaging.

Table II is an example of the results obtained.

Table II.

cc. $N/10$ $KMnO_4$ *required for* 20 cc. *milk.*

Dialysed milk (16 hours).

	Whole milk	Fresh	Pasteurised	Boiled
1	13·10	10·62	11·15	11·30
2	11·80	10·12	10·30	10·55
3	11·08	9·28	9·48	9·70
Av.	11·99	10·01	10·31	10·54
Loss of CaO as % of total		16·70	14·20	12·30
Loss % per hour		1·04	0·89	0·77

Each $KMnO_4$ reading is the average of three.

Fig. 1 shows that at every interval the percentage loss of CaO from fresh milk was greater than from pasteurised and still greater than from boiled.

The differences in loss per cent. of the total CaO between fresh and heated milks at each interval are given below:

Hours' dialysis ...	8	16	24	32	40	48
Fresh—pasteurised	2·6	2·5	3·3	6·0	2·1	8·4
Fresh—boiled	6·4	4·4	4·5	10·7	4·5	12·5

Fig. 2 shows that the rate of loss of soluble CaO agrees with theory until after 32 hours, when the curves rise up to 40 hours and then fall slightly to 48 hours. The more marked rise and fall in the case of the heated milks suggests the intervention of the solvent action of CO_2 on the colloidal calcium, there being, as will be pointed out later, very probably more colloidal calcium in the heated milks. For example, on two occasions a slight precipitate of caseinogen was noticed in the fresh milk after 48 hours' dialysis, but never in the heated milks. Experience having shown that the method was not accurate enough to demonstrate significant losses after 4 hours' dialysis, no readings were attempted at intervals of 28 and 36 hours where, if they had been possible, they would have had a special significance in view of the commencing action of dissolved CO_2.

Exp. 4. Δ *of milk.* The average readings of the Beckmann thermometer for six samples of milk were: fresh 5·502°, pasteurised 5·494°, boiled 5·487°. The readings for pasteurised milk were higher in two cases by 0·001° and

0·002°, than for the corresponding fresh samples. The boiled samples invariably gave smaller depressions than the fresh. The results show that heating progressively reduces the numbers of free ions and of molecules in solution in milk.

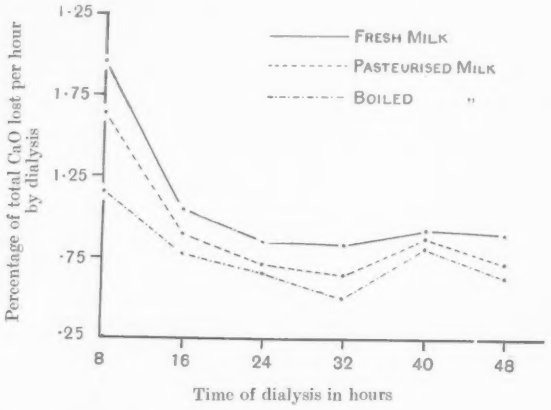

Fig. 2.

Exp. 5. *Effect of heat on the* p_H *of milk.* Milroy [1915] showed that the p_H of milk is lowered by boiling for an hour. His average figures for six samples were: fresh 6·73, boiled 6·59. We determined the p_H in fresh and boiled milks by a modification of the colorimetric method of Kramer and Greene [1921]. Our average results for six samples were: fresh 6·69, boiled 6·55, *i.e.* as in Milroy's experiments a lowering of the p_H by 0·14. The method was not considered sufficiently accurate to bring out the change in p_H after pasteurisation. Milroy suggested that the increase in $H^{\cdot}$ was due to the separation of $Ca_3(PO_4)_2$: thus $3CaHPO_4 \longrightarrow Ca_3(PO_4)_2 + H_3PO_4$.

If the $Ca_3(PO_4)_2$ were separated as a precipitate it should be possible to show a loss of calcium in heated milk by filtration or by centrifuging. He showed an average loss of total CaO of 12·8 % after boiling for an hour and then filtering.

Exp. 6. *Filtration.* Table III shows that almost as much CaO was removed from the fresh as from the heated milks by filtration through paper. The slightly greater losses in the latter could be accounted for by greater volume of colloidal particles; but the possibility of adsorption of ionised proteins by filter paper as shown by Christiansen [1912] is worthy of consideration in this connection. In either case, these results when compared with those of Exps. 2 and 3, are opposed to the idea of the separation of $Ca_3(PO_4)_2$ as an amorphous or crystalline precipitate in heated milk. The results of the next experiment point in the same direction.

Table III.

cc. $N/10$ $KMnO_4$ *for* 20 cc. *milk.*

	Unfiltered	Filtered		
	Fresh	Fresh	Pasteurised	Boiled
1	12·80	12·50	12·60	12·60
2	13·10	12·85	13·10	12·60
3	12·35	12·25	12·20	12·20
4	13·30	13·30	13·20	13·10
5	12·75	12·75	12·50	12·35
6	12·05	12·00	11·90	11·50
7	13·15	13·00	12·95	12·70
8	12·05	12·05	11·95	11·95
9	11·95	11·90	11·85	11·60
Av.	12·61	12·51	12·47	12·29
% CaO	0·1766	0·1752	0·1746	0·1721
Loss as % of total	—	0·8	1·1	2·5

Exp. 7. *Centrifuging.* 50 cc. of milk were centrifuged for from 5 to 30 minutes and the CaO content of the supernatant fluid determined by analysing 20 cc. The experiments were done in duplicate.

Table IV.

cc. $N/10$ $KMnO_4$ *required for* 20 cc. *milk.*

	Time of centrifuging (mins.)	Non-centrifuged	Centrifuged		
		Fresh	Fresh	Pasteurised	Boiled
1	30	11·85	10·85	10·92	10·95
2	30	12·00	11·12	11·15	11·17
3	30	11·85	11·05	10·90	10·92
4	15	11·35	10·88	10·92	10·77
5	5	12·00	11·80	11·82	11·72
Av.		11·81	11·14	11·14	11·11
CaO %		0·1654	0·1560	0·1560	0·1555
Loss as % of total		—	5·7	5·7	5·9

The results show that the losses of CaO were practically identical in all three cases and thus tend to confirm the opinion expressed above. The deposit had similar microscopic appearances in all cases and consisted in greater part of a gelatinous mass containing some leucocytes, bacteria and epithelial cells. Crystals and anything resembling an amorphous deposit were absent. If, then, amorphous or crystalline $Ca_3(PO_4)_2$ is formed by heating milk, not only would it be detected in the deposit after centrifuging but losses proportionate to the amount formed would be detected in the calcium content of the heated milks after filtration or centrifuging. It therefore looks as if the disappearance of soluble calcium after heating is due to its being changed into colloidal form. This idea suggested investigation of the effect of heat on some of the physical properties of milk for which the colloids are chiefly responsible.

Exp. 8. *Viscosity.* Table V shows the relative viscosities (η) of ten samples of milk at 26°.

All the samples of boiled milk showed higher viscosities than the corresponding fresh samples. Except for Nos. 1 and 6 all the samples of pasteurised milk showed slightly lower viscosities than the fresh samples. The reason for

this varying effect of heat on the milk was not clear and further experiments bearing on the relation of the caseinogen to the continuous phase were undertaken.

Table V.

	Fresh	Pasteurised	Boiled
1	1·736	1·745	1·958
2	1·747	1·688	1·902
3	1·725	1·721	1·880
4	1·589	1·555	1·692
5	1·552	1·527	1·613
6	1·584	1·601	1·769
7	1·566	1·521	1·634
8	1·563	1·528	1·661
9	1·530	1·512	1·561
10	1·514	1·506	1·662
Av.	1·610	1·590	1·735

Exp. 9. *Swelling.* 20 cc. each of fresh, pasteurised and boiled milk were clotted as in Exp. 2 and centrifuged for 30 minutes. The curd was then dried on filter paper and its volume determined by displacement.

Table VI.

Volume of coagulum, cc.

	Fresh	Pasteurised	Boiled
1	3·20	3·40	4·20
2	2·20	2·20	3·40
3	2·10	2·30	3·60
4	2·25	1·80	4·20
5	1·90	1·90	3·40
6	2·10	2·10	5·40
7	1·55	2·10	3·00
Av.	2·19	2·26	3·89

The results (Table VI) show that in every sample the volume of curd from boiled milk was much greater than that from the corresponding fresh milk. In sample 4 the volume of curd from the pasteurised milk was less than, but in every other sample either equal to, or greater than, the volume from the corresponding fresh milk. The averages show an increase in swelling, of casein from 20 cc. of milk, of 0·07 cc. after pasteurising and of 1·70 cc. after boiling. The increase of 0·07 cc., however, falls within the limits of accuracy of the measurements, 0·25 cc., and must be regarded therefore as of no significance. We may conclude then that boiling for an hour increases the volume of the casein particles; but that the method employed showed no significant change in volume after pasteurising for 30 minutes. On account of the fall in the viscosity of pasteurised milk one would have expected a corresponding fall in the volume of the casein since the viscosity of protein solutions "is primarily a function of the relative volume occupied by the protein in solutions" [Loeb, 1922].

Hardy [1905] and others have shown that the viscosity of protein salts varies directly as the degree of ionisation and Pauli [1922, p. 29] has shown that, when ionisation is at a maximum, precipitation by alcohol is at a minimum

and *vice versa*. It therefore seemed of interest to make some experiments on the precipitation of milk proteins.

Exp. 10. *Precipitation of colloids.* A solution of 10 % colloidal ferric hydroxide was run into 5 cc. of milk diluted with 5 cc. of water until a clear fluid was obtained after filtration. The flask was shaken constantly during the addition, which occupied a minute, and for 1 minute after and its contents were then filtered. The limit of accuracy was 0·30 cc. of colloidal ferric hydroxide solution.

Table VII.

cc. *of* 10 % *colloidal ferric hydroxide for* 5 cc. *milk.*

	Fresh	Pasteurised	Boiled
1	17·5	16·0	16·0
2	21·7	19·8	19·8
3	20·5	18·85	18·85
4	22·6	—	19·4
5	26·6	26·6	26·0
6	25·0	25·0	24·5
7	25·0	23·7	23·6
Av.	22·7	21·66	21·16

The amounts required for every sample of fresh milk were greater than for the corresponding boiled milk and for every sample of pasteurised milk except 5 and 6. On the average, fresh milk required 1·04 cc. more than pasteurised and 1·5 cc. more than boiled milk for 5 cc. of each, thus indicating a diminution of stability as a result of dehydration or decrease of charge of the colloid. These results do not fit in with Pauli's views which associate minimum viscosity and maximum of precipitation with the isoelectric point, or the point where the number of neutral particles is a maximum.

Exp. 11. *Digestibility of fresh and heated milk.* Liquor Trypsin Co. (A. & H.)[1] was brought to a p_H of 8·1 by addition of Na_2CO_3 and activated by incubating for 12 hours. 2 cc. of activated trypsin were added to 20 cc. of milk and a few drops of $CHCl_3$ and the whole incubated for 1 to 20 hours. The proteins were precipitated with colloidal ferric hydroxide or hot alcohol and aliquot portions of the clear filtrate titrated with $N/20$ NaOH for free acids and for amino-groups by Sörensen's method. Many trials of the method were made, but in most cases the differences observed fell within the experimental error of the method as the results show:

Table VIII.

cc. $N/20$ NaOH *for* 4 cc. *digested milk.*

Hours' digestion	Fresh		Pasteurised		Boiled	
	Free acid	Amino-acid	Free acid	Amino-acid	Free acid	Amino-acid
20	4·00	7·60	3·70	7·40	—	—
4	3·03	3·96	2·67	3·76	2·07	3·78
4	2·60	4·13	2·66	4·12	2·55	3·92
4	1·87	2·00	2·12	2·20	2·40	2·30

[1] We are indebted to Mr Rimington of Emmanuel College, Cambridge, for advice on the most suitable trypsin preparation and on some particulars regarding its use.

It appears therefore, as found by Ellenberger and Hoffmeister [1890], that previous heating of milk has no appreciable effect on its digestibility by proteolytic enzymes or else that our method was not sufficiently accurate. Measurements of viscosity before and after digestion as used by Christiansen [1912] or of conductivity would in our opinion be much more accurate.

Discussion.

The 14 % loss of soluble CaO found in Exp. 1 after pasteurising is not substantiated in Exps. 2 and 3. The discrepancy is probably to be explained by the chemical properties of colloidal ferric hydroxide, but the results serve to emphasise the change in the state of the soluble CaO by heat.

The amounts of soluble CaO obtained in Exp. 2 are very probably a little low on account of adsorption by the coagulum. We believe the percentage losses in total CaO after 32 hours' dialysis are the nearest approximation to the true amounts of diffusible CaO present in the original milk. These figures are: fresh milk 26·4 %, pasteurised 20·4 %, boiled 15·7 %. Very probably the solvent action of CO_2 became effective between 32 and 40 hours and caused the washing out of colloidal calcium as suggested. It is of interest to compare these results with those of other workers. Rona and Michaelis [1909] found 44 % of the CaO in soluble form in fresh cow's milk; Grosser [1913] 23·4 % and after boiling 15 minutes 18 %. Wha [1923] found 50 % in fresh cow's milk and Bell [1925] 23·8 % and after heating for 30 minutes at 77° 19·2 %. Bosworth and Van Slyke [1916] found 30·3 % in fresh goat's milk. Our figure for fresh milk, 26·4 %, agrees fairly well with those of Grosser and Bell and the loss we found after pasteurisation, 6 %, is similar to theirs, viz. 5·4 % and 4·6 % respectively. The heat treatment was not, however, the same in the three cases. We are not aware of any analogous results with which to compare the loss of soluble CaO of 10·7 % after boiling for an hour.

It has been seen that our results oppose the suggestion that the change in p_H after heating is a consequence of the separation of $Ca_3(PO_4)_2$ as a precipitate. It looks therefore as if the loss of soluble Ca was due to its partial transformation into colloidal form; whether as $Ca_3(PO_4)_2$ by itself or as a further combination of Ca with caseinogen, it is difficult to say. Evidence on the effect of heat on the soluble P_2O_5 of cow's milk would help towards a solution. The only data available are those of Grosser and of Bell, which show respectively losses of 0·4 % and 2·8 % of the total P_2O_5 after heating. These losses appear very small when compared with the corresponding losses in soluble CaO, viz. 5·4 % and 4·6 % respectively; but when calculated as per 100 cc. of milk, Bell actually found greater losses of P_2O_5 than of CaO. Thus after heating at 77° for 30 minutes, the losses per 100 cc. of milk were CaO 0·0053 g., P_2O_5 0·0059 g. It appears very probable therefore that heat changes some of the soluble $CaHPO_4$ of milk into $Ca_3(PO_4)_2$ in colloidal form in such a state of subdivision as to be irremovable by filtration or by centrifuging.

It has been abundantly shown that the addition of neutral salts to protein compounds has a depressing effect on the viscosity and swelling of colloidal particles, and that the ion of the salt carrying a charge opposite in sign to the protein ion is responsible. Consequently, the removal of Ca from solution in milk would increase its viscosity and the size of its colloidal particles. The viscosity of milk would also be increased by a decrease in the lyophilic properties of the colloids in the same way as Loeb [1922, p. 252] demonstrated in a solution of egg-albumin after heating. Such an increase in the number of colloidal micellae, or perhaps their appearance for the first time, would lead to the setting up of a Donnan equilibrium whereby water would be attracted into the micellae by the osmotic pressure of the inorganic ions. In this way the relative volume of the colloids would increase and their stability decrease.

Our findings in regard to boiled milk fit in with these suggestions, but not entirely in regard to pasteurised milk, for the viscosity was slightly lowered and the relative volume of the casein scarcely affected by pasteurisation. These discrepancies indicate the need of further study of the effects of heat varying in degree and duration on the physical properties of milk. Possibly other changes and other factors not mentioned would be shown to be of importance.

SUMMARY.

1. When the proteins were precipitated with colloidal ferric hydroxide 85 % of the total CaO was found in solution in the case of whole fresh milk and 71 % in that of whole milk heated for 30 minutes at 65°.

2. About 26 % of the total CaO was found in diffusible form in fresh milk, about 20 % in pasteurised milk and about 15 % in boiled milk. The losses in soluble CaO after heating were believed to be due to the formation of colloidal $Ca_3(PO_4)_2$ from the soluble $CaHPO_4$, which would account for the increase in acidity observed. Precipitation of amorphous or crystalline $Ca_3(PO_4)_2$ by heat was not detected.

3. Heat progressively lessened the stability of the colloids and increased the size of the protein particles. Pasteurisation for 30 minutes caused a slight fall in viscosity, but boiling for an hour a marked increase. The mechanisms by which heat could cause these various effects are discussed.

4. No differences were observed in the rates of tryptic digestion of milk before and after heating.

We desire to express our thanks to Dr J. B. Orr who suggested the research, and to Dr W. Thomas, University of Aberdeen, for advice and criticism.

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CXI. STUDIES ON THE EFFECT OF HEAT ON MILK.

II. THE INFLUENCE OF DIETS OF FRESH AND TREATED COW'S MILK ON THE CALCIUM, PHOSPHORUS AND NITROGEN METABOLISM OF THE YOUNG PIG.

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In the first paper of this series [Magee and Harvey, 1926] we reported, as a result of heating milk, a progressive fall in the diffusible calcium. On the other hand, several workers have demonstrated that the souring of milk causes a rise in the diffusible calcium [Grosser, 1913; Van Slyke and Bosworth, 1916; Wha, 1923] and phosphorus [György, 1923]. It seemed of interest, therefore, to investigate the effect of diets of raw, heated and sour milk on the metabolism of calcium and phosphorus in young animals. Study of the metabolism of nitrogen was also considered desirable; firstly because we observed that heat affected the physical state of the colloids, but not the digestibility of the proteins, and secondly because the lactic acid of sour milk precipitates the caseinogen as a base-free protein [György, 1923].

The most recent review of the literature available [Lane-Claypon, 1916] would lead one to conclude that infants fed on heated cow's milk thrive better than on raw milk. The later experiments of Daniels and Loughlin [1920] and Daniels and Stearns [1924] point to a contrary conclusion. The former attributed the defective nutrition they observed in rats fed on heated milk to loss of calcium by precipitation. The latter found that infants fed on very rapidly boiled milk stored more calcium and phosphorus than on milk pasteurised for half an hour. They contend that the extent to which milk is detrimentally affected by heat depends more on the duration of the heating than on the temperature.

Experimental.

Pigs were used for these experiments. They are more suitable animals for metabolism experiments than calves and, on account of their rapid rate of growth, errors of diet soon become apparent. Cow's milk, however, is not well suited for the requirements of the young pig. It contains on an average

0·16 % CaO and 0·24 % P_2O_5, *i.e.* CaO : P_2O_5 = 1 : 1·5, whereas sow's milk, the natural food of the suckling pig, contains 0·39 % CaO and 0·36 % P_2O_5, *i.e.* CaO : P_2O_5 = 1 : 0·92. It is impossible to supply the food requirements of pigs of the age employed with cow's milk only. Cereals had therefore to be added to their ration. These raised the energy and phosphorus contents, but had little effect on the calcium, so that in regard to the CaO : P_2O_5 ratio the diet was rendered still more unsuitable. We decided, nevertheless, as a preliminary to further experiments, to try the effect of feeding a ration of cereals with heated, fresh or sour milk on the calcium, phosphorus and nitrogen metabolism of a young pig. The CaO : P_2O_5 ratio of the intake was 1 : 3 and the periods were in the above order. The animal broke down after a month and signs of rickets appeared. The retention of CaO fell steadily until finally only 4 % of the intake was being absorbed as compared with 28 % at the beginning. The rate of retention of N was, however, maintained and indeed increased.

This preliminary experiment showed that the ration of cow's milk and cereals was inadequate for proper growth of a young pig, owing most probably to its low calcium content; for Henderson [1925] has shown that a diet with a CaO : P_2O_5 ratio of 1 : 2·5 caused defective bone formation in a young pig in the absence of ultra-violet light. It was, therefore, necessary in the subsequent experiments to supplement the diet of milk and cereals with calcium.

Exp. 1. *Animals.* Two hog pigs aged 12 weeks, No. 1 weighing 21 kg. and No. 2 16 kg.

Diets per day. Basal rations. Pig 1. Oatmeal, maize and sharps 220 g. each, blood meal 30 g., olive oil 5 cc., $CaCl_2$ solution 100 cc. (5·621 g. CaO) and some saccharin to sweeten. Pig. 2. three-fourths, *i.e.* approximately 16/21, of the ration fed to pig 1. The CaO : P_2O_5 ratio of the diet when milk was introduced was approximately 1 : 1·2. In regard to the proper ratio for pigs of the age employed, we are still somewhat in the dark, for while a ratio of 1 : 0·92 is proper for a suckling pig, it does not follow that it is so for one 10 or 12 weeks old. The adjustment made must be regarded, therefore, as only approximate.

Periods. (1) Basal ration period—8 days. The above basal ration was fed in order to determine the nature of the balance before milk was introduced.

(2), (3) and (4) Pasteurised, Fresh and Sour Milk periods, each 12 days. 1500 cc. daily of the appropriate milk were given to pig 1 and 1125 to pig 2. For the latter the order was: Fresh, Pasteurised and Sour Periods.

(5) Post-period—6 days. The $CaCl_2$ supplement was reduced by half in order to determine whether big reductions in the intake of soluble CaO would accentuate the effects induced by feeding heated milk. Pig 1 got 1500 cc. fresh milk and pig 2, 1125 cc. per day.

Methods. The methods of collection of excreta and of analyses were similar to those in general use at this Institute [Husband, Godden and Richards, 1923]. Excreta were collected and analysed every 48 hours. At the beginning of each period an interval of 48 hours was allowed to elapse before analyses were begun.

The milk was all from the same cow, one of the Institute herd (the bacterial count of pooled samples of Institute milk at the time of the experiments was found to be between 2000 and 4000 per cc.). The intake of CaO, P_2O_5 and N was determined by previous analyses of the cereals and by drawing off daily suitable quantities of milk from the bulk fed to the pigs and analysing the composite sample at short intervals. "Pasteurised" milk was prepared by keeping a bulk of fresh milk at 70° in a vessel immersed in a water-bath for 30 minutes. The film was removed after cooling rapidly. "Sour" milk was prepared by inoculating the requisite amount of fresh milk for the daily ration with a pure culture of *B. bulgaricus* and incubating 24 hours. The sample for analysis was taken from the bulk of fresh milk, not from the sour milk.

The cereal part of the ration mixed with sufficient milk was all given at the morning feed, the remainder of the milk, if fresh or pasteurised, was kept cool and some formaldehyde added so as to preserve it until the evening feed.

Results. The results are shown in the figure.

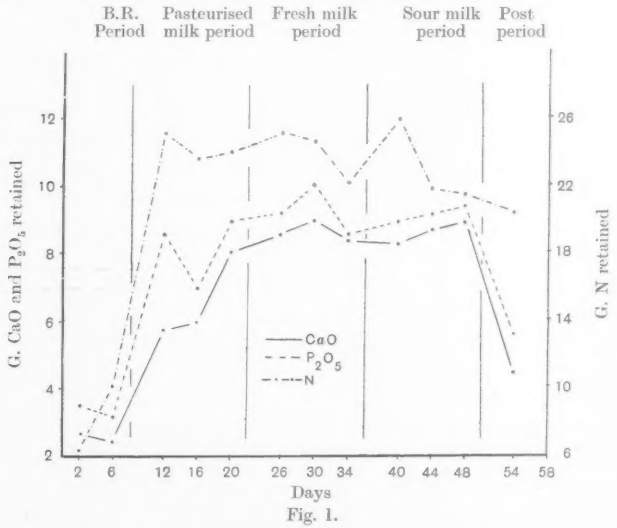

Fig. 1.

The 48-hourly balances when plotted gave curves with a great number of small oscillations. In order to smooth these out an average was taken of consecutive pairs of values in each period and *graphed*. In the post-period the three values were taken together and averaged.

In pig 1 (Fig. 1) it is seen that on the addition of milk to the basal ration there is a marked increase in the retention of CaO and P_2O_5 which is continued when fresh milk is substituted for pasteurised. During the latter part of the fresh period the retention falls slightly but increases steadily when sour milk is substituted for fresh. The N curve does not suggest any definite conclusions. In view of the results shown in the N graph of pig 2 (Fig. 2) it appears

probable that pig 1 was on a luxus consumption of N throughout the three milk periods. The initial increases in the balance on changing from pasteurised milk to fresh and from fresh to sour seem to support this view.

In pig 2 the retention of CaO and P_2O_5 rises steadily in the fresh period, but falls in the pasteurised and rises again in the sour period. The retention of N, on the whole, follows that of CaO and P_2O_5. It will be noted, however, that on the introduction of milk the retention of N in both pigs, unlike that of CaO and P_2O_5, rises rapidly.

In the post-period in both pigs (when the $CaCl_2$ supplement was reduced by half) the retention of CaO, P_2O_5 and N falls markedly. It is interesting to note that the diminished balances of P_2O_5 and N were largely due to increased urinary excretion, as is shown in Table I.

Table I. *Urinary excretion.*

(Average in g. per 2 days.)

Pig 1

Period	Basal	Past.	Fresh	Sour	Post
CaO	1·23	1·09	0·85	0·72	0·20
P_2O_5	0·44	0·68	0·66	1·17	5·33
N	17·36	17·82	19·47	20·31	23·26

Pig 2

Period	Basal	Fresh	Past.	Sour	Post
CaO	1·17	1·12	0·70	0·78	0·29
P_2O_5	0·32	0·62	0·71	0·77	4·04
N	13·65	16·74	18·50	18·50	19·88

The P_2O_5 excretion was increased about fivefold. The next highest value for the urinary P_2O_5 was in the sour period. This supports the finding of Blatherwick and Long [1922] that sour milk ingestion increased the urinary phosphorus.

This experiment seemed to indicate that the retention of CaO, P_2O_5 and N was lower on the ration of pasteurised milk than on that of fresh or sour milk. The next step in the investigation was to try the effect of adding enough soluble calcium to the diet to make good the loss due to pasteurising.

Exp. 2. *Animals.* Two hog pigs aged 15 weeks and weighing 25 kg. each.

Periods. Pig 1—1st fresh 15 days: pasteurised plus $CaCl_2$ 15: 2nd fresh 12.

Pig. 2—Pasteurised plus $CaCl_2$ 15 days: fresh 15: boiled 12.

Diets per day. Oatmeal, maize and sharps 250 g. each, blood meal 30 g., milk 1500 cc., olive oil 10 cc., $CaCl_2$ solution 75 cc. (4·043 g. CaO) and some saccharin. During the pasteurised plus $CaCl_2$ period 81 cc. $CaCl_2$ solution (4·366 g. CaO) were given. The extra supplement of $CaCl_2$ was equivalent to about double the loss in diffusible CaO after pasteurising as estimated from the data of our earlier experiments [Magee and Harvey, 1926]. The CaO : P_2O_5 ratio of the diet was approximately 1 : 1·6.

The technique was in general the same as in Exp. 1. The boiled milk was prepared by keeping the water in the outside vessel boiling vigorously for an hour: pasteurising was done at 70° for 30 minutes.

Excreta were collected and analysed every 72 hours. An interval of 72

hours was allowed to elapse at the beginning of each period before analyses were begun.

Results. The results are shown in Table II.

Table II.

(Average in g. per 3 days.)

	Pig 1			Pig 2		
Period	1st fresh	Past. + $CaCl_2$	2nd fresh	Past. + $CaCl_2$	Fresh	Boiled
Balance.						
CaO	11·25	13·35	12·57	13·07	11·37	11·94
P_2O_5	14·76	16·21	13·95	15·85	13·71	12·70
N	32·81	33·39	29·64	41·50	32·79	28·91
Urinary excretion.						
CaO	1·31	1·36	1·24	0·65	0·63	0·45
P_2O_5	5·52	5·28	6·29	3·91	5·38	5·52
N	43·33	41·44	48·88	34·96	38·84	47·60

As in Exp. 1 the retention of CaO and P_2O_5 did not rise to a steady level until about the 9th day of the first milk period. The first three values in this period were therefore discarded and an average made of the other two. This average represented fairly accurately the equilibrium state. The values in each of the other periods and, in the case of N, in all the periods were obtained by averaging all the results in each. Thus, the results were expressed in tabular form which was to be desired; for, owing to there being five values in each of the first two periods, averaging of consecutive pairs to smooth out the graphs as in Exp. 1 was not possible.

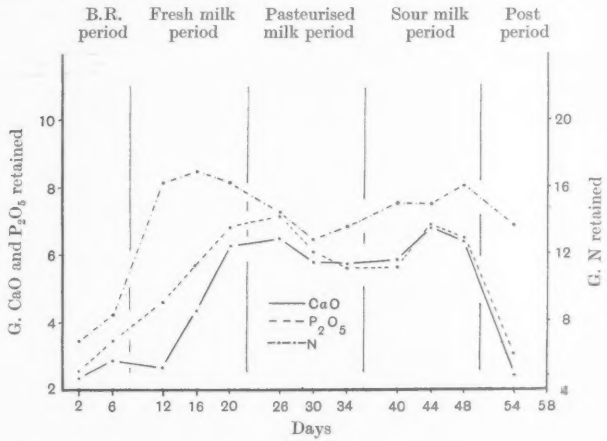

Fig. 2.

It will be seen from the table that the retention of CaO, P_2O_5 and N was highest in the pasteurised plus $CaCl_2$ period and that the extra supplement of $CaCl_2$ caused an increase in the retention of CaO equal to more than twice the amount added (0·323 g. CaO). The retention of P_2O_5 and N was lower in the

boiled period than in the fresh, but the retention of CaO was slightly higher in the former than in the latter. This finding is not in conformity with the other results either of this experiment or of the previous one. Very probably the retention was to some extent apparent rather than real, for there was a tendency to constipation during the boiled period. The low urinary CaO in this period would seem to substantiate this view.

As in the post-period of Exp. 1 reduction of the soluble CaO of the intake caused a rise in the urinary P_2O_5 and N. Thus the excretion is lowest in the pasteurised plus $CaCl_2$ period and highest in the boiled period (pig 2).

This experiment suggested that the detrimental effect of heat on milk could be removed, in so far as the retention of CaO, P_2O_5 and N is concerned, by the addition of calcium in solution. Exp. 1 shows, however, that there was practically no difference between the retention of CaO, P_2O_5 and N on the fresh and on the sour milk ration, though the soluble CaO content of the latter was greater than that of the former. There is no apparent reason for this difference.

It would appear from this investigation that the retention of calcium, phosphorus and nitrogen by young animals is lower on a diet containing heated milk than on one containing fresh or sour milk, and further that the addition of soluble calcium to the heated milk ration increases the amount of calcium, phosphorus and nitrogen retained by more than the weight of the calcium added. The changes in the state of the colloids, described in our first paper, had evidently no effect on the assimilation of nitrogen. It is therefore suggested that the reduction in the soluble calcium by heat is an important factor in the lowering of the retention of calcium, phosphorus and nitrogen of young animals fed on heated milk as compared with raw milk.

There are no data in these experiments that would throw any light on the effect on metabolism of alteration by heat in other milk constituents of known or unknown constitution, so that discussion along these lines is not possible.

Summary.

1. A young pig kept under experimental conditions on a diet of cow's milk and cereals showed signs of rickets after 30 days. The addition of a soluble calcium salt to the diet of cereals and milk enabled the animals fed on it to remain in a thriving condition for 60 days.

2. On a diet of cereals and milk the retention of calcium, phosphorus and nitrogen was lower with heated milk than with fresh or sour milk.

3. The addition of soluble calcium to a ration containing heated milk raised the retention of calcium, phosphorus and nitrogen by more than the weight of the calcium added.

4. Reduction of the soluble calcium of the diet increased the urinary phosphorus and nitrogen.

5. It is suggested that heat has a detrimental effect on the nutritive value of milk and that one of the important contributing factors is the reduction in the amount of soluble calcium.

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